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Our proposed research plan involves laboratory studies using a SCID mouse model of human metastatic breast cancer, as well as in vitro MTT(3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide) and colony assays, using established breast cancer cell lines, to examine the potency and toxicity of various EGF-Genistein conjugates. In an effort to generate more effective conjugates, we have employed a variety of crosslinking agents and photolysis conditions. Furthermore, we have established HPLC(high performance liquid chromatography) procedures to characterize and isolate the EGF components of the reaction mixture. We have also conjugated EGF to other small molecules which by themselves have been shown to possess anti-cancer activity. The knowledge gained from these studies is expected to lead to more effective biotherapy and combined biochemotherapy regimens for the treatment of breast cancer patients.

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Roland Luther
Principal Investigator Signature

Nov 4, 1999
Date

TABLE OF CONTENTS

INTRODUCTION

We have continued our efforts to optimize the design of the EGF-Genistein and related tyrosine kinase inhibitor conjugates. The goal of these continuing efforts is to prepare a new generation of EGF conjugates with unprecedented activity as well as stability. The design optimization represents work done at the Hughes Institute whereas the mouse and monkey studies are being conducted at the University of Minnesota. The work as well as analyses are ongoing and no conclusions are yet possible as to whether or not the novel EGF conjugates will be superior to the first generation EGF conjugates. Depending on these results, we will pick the most promising conjugate and start its use as part of combined biochemotherapy regimens, as originally proposed in our application.

BODY

SECTION I: DESIGN OPTIMIZATION

MATERIALS AND METHODS

Preparation of EGF-Genistein and Related Conjugates . rhEGF was produced in *E. coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies and the mature protein was recovered by trypsin-cleavage followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of 4.6 ± 0.2. The endotoxin level was 0.17 EU/mg.

The recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) was initially employed in the synthesis of the EGF-Genistein(Gen) conjugates. Sulfo-SANPAH was dissolved in DMSO and used to modify EGF at molar ratios of 1:1 - 1:10, EGF to crosslinker. Following size-exclusion chromatography to remove unreacted crosslinker and small molecular weight reaction products, the modified rhEGF was mixed with a 10:1 or 20:1 molar ratio of Gen (LC Laboratories, Woburn, MA) [50 mM solution in dimethyl sulfoxide (DMSO)] and then irradiated for 10 - 60 min with long-wave UV light (366 nm Model UVGL-58 Mineralight; UVP, Upland, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a molar excess of Genistein resulted in the attachment of Gen to lysine 28, lysine 48, or the N-terminal residue of EGF. Excess Gen in the reaction mixture was removed by passage through a G25-Sephadex prepacked column.

The EGF- Gen conjugate was subsequently filter-sterilized and the protein concentration determined using the Bicinchoninic Acid(BCA) Protein Assay kit obtained from Sigma Chemical Company. Bicinchoninic acid is a chromogenic reagent, highly specific for Cu(I), which forms a purple complex with an absorbance at 562 nm that is directly proportional to the protein concentration.

In addition to Sulfo-SANPAH, we also used the following crosslinking agents obtained from Pierce Chemical Company: N-5-azido-2-nitrobenzoyloxysuccinimide(ANB-NOS), Sulfosuccinimidyl 2-[m-azido-o-nitrobenzamido]ethyl-1,3'-dithiopropionate(SAND), and Sulfosuccinimidyl(perfluoroazidobenzamido)ethyl-1,3-dithiopropionate(SFAD). These crosslinkers are of different chain lengths, ANB-NOS being the shortest at 7.7 A, and all have a phenyl azide at one end

to react with Genistein following photolysis. The other end of the crosslinker contains an N-hyrodroxysuccinimide ester to react with protein amino groups. SAND and SFAD are cleavable by thiols.

To avoid exposing EGF to the possible harmful effects of UV light, we have also photolyzed the crosslinker-Genistein mixture prior to the addition of EGF. We dissolved both the crosslinker and Genistein in DMSO and mixed them together using a 20:1, 10:1, 5:1, or 2.5:1 molar ratio of Genistein to crosslinker. Photolysis was performed at room temperature for periods of time from 15 minutes to 48 hr using either a Model UVM-57(302 nm mid-range wavelength) or Model UVGL-58(366 nm longwave) UV lamp from UVP(Upland, CA). Following photolysis, the mixture was added to a solution of EGF(in PBS) at molar ratios of 2:1 to 10:1, crosslinker:EGF in a maximum final DMSO concentration of 10%.

In an effort to generate more potent EGF conjugates, we have also attempted to link other compounds which have themselves been shown to possess cytotoxic activity in in vitro systems. These compounds include two Genistein analogues, DDE24 and DDE41, which have been modified to contain an N-hydroxysuccinimide ester for direct conjugation to EGF in the absence of photolysis. We have also employed the above photolysis procedures to form EGF conjugates of the novel quinazoline derivatives, WHI-P97 and WHI-P154, as well as of DDE24 and DDE41.

HPLC Analysis. Reverse phase HPLC using a Hewlett-Packard (HP) 1100 series HPLC instrument was used to monitor and characterize the EGF-Gen conjugations. Analytical HPLC was performed using a LiChrospher 100(RP-18, 5 um) reverse phase column (250x4 mm, Hewlett-Packard). HPLC chromatograms were run at wavelengths of 220 nm, 280 nm, 308 nm, or 480 nm using the multiple wavelength detector option supplied with the instrument. UV spectra were generated for the individual peaks of

interest in the chromatogram. Five - 100 uL samples were applied to the above column and analysis was achieved using a gradient flow as follows: t = 0, 20% D; t = 5, 30% D; t = 9, 38% D; t - 20, 43.5% D; t = 35, 100% D; t - 50, 100% D; t = 55, 20% D. Eluent A consisted of a mixture of 0.1% trifluoroacetic acid(TFA) in water and eluent D contained 80% acetonitrile (CH3CN), 20% H_2O, and 0.1% TFA.

Size-exclusion chromatography was carried out using a Beckman System Gold Instrument equipped with either a preparative TSKG3000SW column equilibrated in 100 mM sodium phosphate buffer, pH 6.8 at a flow rate of 3 mL/minute or an analytical TSKG3000PW column run in the same buffer at a flow rate of 0.2 mL/min

Mass Spectrometry. Mass spectrometric analysis was routinely performed to determine the relative molecular weights of the modified EGF and EGF-Genistein conjugates using a Hewlett-Packard Model G2025A matrix-assisted laser desorption/ionization mass spectrometer with linear time-of-flight mode (MALDI-TOF). In conjunction with the Hewlett-Packard instrument were a sample preparation assembly model G2024A including a high vacuum pump and a Dos-Chem station controller model G1030A. Before starting the experiment, the instrument was calibrated with protein standards G2025A supplied by Hewlett-Packard; mass calibration was used by peak centroiding at the 80% level. Sinnapinic acid(Hewlett-Packard) was used as a matrix source. Samples were prepared by spotting 1 uL of a mixture of protein, in phosphate buffer, with the matrix solution(1:1, v/v) on the gold surface of the probe with subsequent evaporation under vacuum. Ionization was accomplished with a laser radiating at a 337-nm wavelength(5 ns pulses, laser energy 1.97 uJ) in both single shot and multiple shot modes. The analyzer was used in the linear mode at an accelerating voltage of 28 kV. The obtained spectra represent the sum of consecutive laser shots and have not been smoothed.

SDS-PAGE Analysis. SDS-PAGE was used to monitor the preparation and purification of the EGF-Genistein conjugates. 10 - 20% tris tricine gradient gels (BioRad Laboratories) were stained with GelCode Blue to visualize the protein bands.

Breast Cancer Cells. MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. BT-20 (ATCC HTB-19) is another EGF-R positive breast cancer cell line isolated from the primary breast tumor of a 74 year old patient with grade II mammary adenocarcinoma. SK-BR-3(ATCC HTB-30) is an adenocarcinoma of the mammary gland which was isolated from the pleural effusion of a 43 year old female; SQ-20B is a squamous cell carcinoma of the head and neck.

MDA-MB-231 cells are cultured in Leibovitz's L-15 medium plus glutamine; BT-20 breast cancer cells are maintained in MEM medium containing 0.1 mM NEAA and Earle's BSS; SK-BR-3 cells are cultured in McCoy's medium and SQ-20B in DMEM. All media are further supplemented with 10 % fetal bovine serum(DMEM contains 20% FBS, not heat-inactivated). For subculturing, medium is removed from the flasks containing a confluent layer of cells and fresh 0.25% trypsin added for 1-2 min. Trypsin is removed and cultures incubated for 5-10 min at 37°C until the cells detached. Fresh medium is then added and the cells aspirated and dispensed into new flasks.

Cytotoxic Activity of EGF-Genistein and Related EGFConjugates. The specific cytotoxic activity of the EGF-Genistein conjugates is determined initially using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim Corp., Indianapolis, IN). Briefly, exponentially growing breast cancer cells are seeded into a 96-well plate at a density of 2.0×10^4 cells/well and incubated for 18 - 24 hr at 37°C prior

to drug exposure. On the day of treatment, culture medium is carefully aspirated from the wells and replaced with fresh medium containing the EGF-Genistein conjugates or unconjugated EGF. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 48 - 72 hours at 37°C in a humidified 5% CO_2 atmosphere(MDA-MB-231 cells are incubated in the absence of CO_2). To each well, 10 µl of MTT (0.5 mg/ml final concentration) was added and the plates incubated at 37°C for 4 hours to allow MTT to form formazan crystals by reacting with metabolically active cells . The formazan crystals were solubilized for a minimum of 4 hr at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well is measured in a microplate reader (Labsystems) at 540 nm. The absorbance is a measure of cell viability; the greater the absorbance the greater the cell viability.

Colony Assays. After overnight treatment with EGF-Gen or PBS, cells were resuspended in clonogenic medium consisting of alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/mL/dish and cultured in a humidified 5% CO2 incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: % Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100.

Zebra Fish Embryo Test System. Zebra fish embryos were incubated with EGF-Genistein conjugates and observed for inhibition of cell division and embryonic development. Dechorionated embryos, at the 2 - 4 cell stage, were exposed to the drugs in 24-well plates and incubated at a constant temperature of 82° F. Various concentrations of the conjugates were added to the embryonic medium in a toal volume of 500 uL and the embryos observed for 30 minutes - 3 hr.

RESULTS AND DISCUSSION

Our initial EGF-Genistein conjugates were formed using Sulfo-SANPAH as the photolabile crosslinker. EGF was modified using a 10:1 molar ratio of Sulfo-SANPAH : EGF followed by 60 minutes of photolysis in the presence of longwave UV and a 10 - 20-fold molar excess of Genistein. Size-exclusion HPLC revealed the presence of high-molecular weight material and SDS - PAGE showed the presence of EGF multimers. We also noted that this EGF conjugate precipitated out of solution during short-term storage at 4° C or when frozen for longer periods of time further reducing the yield of the active EGF - Gen conjugate.

Photolyzing the highly SANPAH-modified EGF at high protein concentrations appeared to be causing the formation of EGF-EGF multimers and denaturing the EGF so we carried out photolysis on the Sulfo-SANPAH-Genistein mixture(in DMSO) prior to the addition of the EGF. This "pre-photolysis" mixture contained a 10:1 or 2.5:1 molar excess of Genistein to crosslinker in order to increase the opportunity for the active nitrene to link to Genistein rather han to another SANPAH or EGF molecule. EGF was added to this mixture following photolysis and unreacted SANPAH and Genistein were removed using G-25 Sephadex column chromatography. A representative analytical size-exclusion HPLC analysis revealed the presence of high molecular weight aggregates eluting from 30 - 45 minutes post-injection (**Figure 1**). Unmodified EGF typically elutes in this system at 50 - 60 minutes.

Reverse-phase HPLC analysis was performed on EGF- Genistein conjugates prepared using a 4:1 ratio of the SANPAH crosslinker to EGF. **Figure 2A** shows the HPLC pattern for the SANPAH-modified EGF itself (in the absence of Genistein), **Figure 2B** shows the pattern for the EGF-Gen

conjugate formed when the SANPAH-modified EGF is photolyzed in the presence of a 10:1 molar ratio of Genistein, and **Figure 2C** shows the pattern for an EGF-Gen conjugate formed by photolyzing the SANPAH and Genistein prior to adding the EGF.

In this series of experiments, EGF had a retention time between 13 and 17 minutes and is detected at wavelengths of 220 and 280 nm. Since there is no detectable absorbance at 480 nm (characteristic of the SANPAH crosslinker), this peak represents unmodified EGF. The UV spectrum shows a peak at 280 nm which is characteristic of aromatic amino acid residues in proteins. All of the HPLC traces show a number of peaks which are detectable at 220, 280, and 480 nm. The UV spectra of these peaks reveal the absorbance peak at 280 nm (characteristic of EGF) as well as an absorbance at 480 nm indicating the presence of the SANPAH moiety.

This reverse-phase system was also used to verify the presence of unreacted EGF in fraction III from the size-exclusion HPLC separation shown as **Figure 1**. **Figure 2D** shows that HPLC fraction III has a retention time of 12.196 minutes and a UV spectrum characteristic of unmodified EGF.

When Genistein has been added to the conjugation mixture (**Figures 2B and 2C**), the presence of unreacted Genistein, with a retention time of 15 - 18 minutes, can be detected at wavelengths of 220 and 280 nm in this reverse-phase system. The UV spectrum is characteristic with a shoulder at 330 nm; UV spectra of potential EGF-Genisten conjugates, eluting at 36 - 38 minutes, possess this shoulder along with an absorbance at 480 nm.

An EGF-Genistein conjugate was made using a prephotolyzed mixture of SANPAH and Genistein with Genistein in a 10:1 molar excess. Photolysis was carried out for 48 hr under longwave UV and the mix added to EGF at a

2:1 molar ratio of crosslinker to EGF. This conjugate was put throught the preparative size-exclusion HPLC and fractions collected for the MTT assay. In this experiment, the so-called "heavy material" in fractions I and III (**Figure 3A**) showed significant inhibition of BT-20 cells in the MTT assay, whereas fraction IV(unreacted EGF) and the unpurified mixture showed no inhibition (**Figure 3B**). It is possible that unmodified EGF possesses a greater affinity for the EGF receptor and could successfully block the binding of the EGF-Gen conjugate.

We also made EGF- 24 and EGF - 41 conjugates by prephotolyzing mixtures containing an excess of DDE24 or DDE41 to SANPAH. Lower ratios were used because these compounds are very insoluble in aqueous solutions. EGF was added after the photolysis but these conjugates were not significantly more effective at inhibiting breast cancer cells than the conjugates prepared by directly linking DDE24 or DDE41 in the absence of photolysis.

We then substituted shorter chain-length and less hydrophobic crosslinkers for SANPAH in order to reduce aggregation due to protein-protein hydrophobic interactions. The short-chain crosslinker, ANB-NOS, results in less precipitation/aggregation than was seen using Sulfo-SANPAH. Since Genistein is relatively insoluble in aqueous solutions, we carried out the pre-photolysis using a 5:1 or 10:1 molar ratio of Genistein to crosslinker and a 5:1 or 10:1 ratio of crosslinker:EGF. EGF-Gen conjugates were prepared by photolyzing ANB-NOS-modified EGF for one hr under longwave UV in the presence of excess Genistein or by prephotolyzing the ANB-NOS-Genistein mixture for 3.25 or 6.25 hr under longwave UV before adding EGF. The final DMSO concentration was maintained at 10%. These conjugates were subsequently tested at concentrations of 25 and 50 ug/mL for their effects on zebra fish embryo

cell division and development. EGF was included in these experiments as a control.

Only the EGF-Gen conjugates made by prephotolyzing the ANB-NOS/Genistein mixture for 6.25 hr showed an effect on embryogenesis. A 10:1 ratio of the ANB-NOS crosslinker to EGF was used; the conjugate containing a 5:1 ratio of Genistein to ANB-NOS in the prephotolysis mix caused lysis of the embryos after one hr incubation at both concentrations. The conjugate prepared with a 10:1 ratio of Genistein in the prephotolysis mix also caused lysis of the embryos within one hr of incubation at the high concentration but required up to two hr to see the same affect at the low concentration. The EGF control showed normal embryo development. **Figure 4** shows representative results of this assay.

We then obtained the SQ-20B and SK-BR-3 cell lines and used them to test a variety of EGF conjugates, including EGF- Gen prepared using the SFAD crosslinker. SFAD-modified EGF was photolyzed in the presence of excess Genistein and SFAD/Genistein mixtures were prephotolyzed for various periods of time using a mid-range UV lamp. We also linked Genistein and P97 (a rationally designed small molecule EGFR inhibitor developed at the Hughes Institute) to EGF using longwave UV and photolysis in the presence of SANPAH-modified EGF. Additional EGF conjugates were prepared from DDE24 and DDE41, either by direct linkage or using ANB-NOS as the crosslinker. **Figures 5A - 5D** show results of MTT assays using these EGF conjugates against these new cell lines. All of the conjugates, as well as the EGF control, exhibit some degree of inibition of these cells lines indicating that breast cancer cell lines vary in their susceptibility to the EGF conjugates.

SECTION II. ANIMAL STUDIES

MATERIALS AND METHODS

The detailed procedures for murine and primate toxicity studies were detailed in the original grant application and also reported in the previously submitted manuscripts regarding the animal toxicity of the first generation EGF conjugates.

RESULTS AND DISCUSSION

I. Toxicity Studies in Monkeys. In our last report, we mentioned that we examined the toxicity of EGF-ANB-NOS-Genistein and EGF-ANB-NOS-DDE41 (EGF-41) in cynomolgus monkeys. Both agents were well tolerated by monkeys. A detailed report of the clinical findings and raw data was enclosed as **Appendix 2** in the last report. The monkeys have been sacrificed and a detailed histopathology report is included in the present report as **Appendix 2**. No evidence of test article-related lesions was found in monkey 68-K treated with a 1 mg i.v. bolus of EGF-41; 68-I treated with a 5 mg i.v. bolus of EGF-41; 68-N treated with a 1 mg i.v. bolus of EGF-ANB-NOS-Genistein; or 68-J treated with a 5 mg i.v. bolus of EGF-ANB-NOS-Genistein.

II. Toxicity Studies in SCID Mice. We examined in a small pilot study the toxicity of combined chemo-biotherapy regimens employing EGF-Genistein plus cytoxan, taxol, methotrexate, or adriamycin in healthy SCID mice. Taxol + EGF-Genistein, Cytoxan + EGF-Genistein as well as Methotrexate + EGF-Genistein combinations were well tolerated. The experimental data are included in **Appendix 3**. More extensive toxicity studies will be performed during the next grant period.

III. Efficacy Studies in SCID Mice. We examined the biologic activity of various chemo-biotherapy regimens in SCID mice xenografted with MDA-MB-231 human breast cancer cells. These regimens utilized EGF-Genistein at a high dose level (100 µg/mouse = 5 mg/kg) and a 4-day treatment schedule **(appendix 4)**. While the combination therapies showed significant anti-cancer activity, no additional benefit was achieved by the combination with EGF-Genistein.

During the next grant period, we will continue our stepwise preclinical development of EGF-Genistein conjugates as a potential new class of anti-breast cancer drugs. The studies will focus both on the conjugation chemistry of novel EGFR tyrosine kinase inhibitors as well as the evaluation of their toxicity, pharmacokinetics, and efficacy in established preclinical animal models as in the previous years.

Appendix I

Figure Legends

Figure 1- Figure 1 shows an example of a size-exclusion HPLC profile of an EGF-Genistein conjugate prepared using a 4:1 ratio of crosslinker to EGF and a prephotolyzed mixture containing a 10:1 molar excess of Genistein to SANPAH. The Beckman System Gold HPLC was equipped with a TSKG3000PW analytical column equilibrated in 100 mM sodium phosphate buffer, pH 6.8, at a flow rate of 0.2 mL/minute. Fractions are labeled I, II, and III.

Figure 2A - Figure 2A shows a reverse-phase HPLC pattern of EGF-SANPAH made using a 4:1 molar ratio of SANPAH to EGF. UV spectra are included for the major peaks; unmodified EGF elutes at 16.864 minutes in this run. The spectrum of the peak eluting at 30.026 is characteristic of the SANPAH crosslinker.

Figure 2B - Figure 2B shows a reverse-phase HPLC pattern of EGF-Genistein made by photolyzing the EGF-SANPAH in the presence of a 10-fold molar excess of Genistein. In addition to the unmodified EGF(retention time of 16.858 min), a peak of unreacted Genistein is also present at 18.042 min. UV spectra are included for representative peaks; the peak eluting at 37.474 min. appears to have characteristics of EGF, SANPAH, and Genistein.

Figure 2C - Figure 2C shows a reverse-phase HPLC pattern of EGF-Genistein made by prephotolyzing the SANPAH/Genistein mixture prior to adding the EGF. The pattern shows peaks characteristic of unmodified EGF and Genistein, as well as of a possible EGF-Genistein conjugate.

Figure 2D - Figure 2D is a reverse-phase HPLC trace of fraction III shown in **Figure 1** . The peak with a retention time of 12.196 min. and a UV spectrum characteristic of unmodified EGF verifies that the size-exclusion chromatography is able to remove a significant amount of the free EGF

remaining in the conjugation mixture.

Figure 3A shows a preparative size-exclusion purification of an EGF-Genistein conjugate prepared using the prephotolyzed SANPAH/Genistein mixture. Fractions were isolated and tested against the BT-20 breast cancer cell line using the MTT assay(**Figure 3B**). In the MTT assay, the greater the response(y-axis), the greater the cell viability.

Figure 4A shows a zebra fish embryo treated with 50 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 5:1 ratio of Genistein to ANB-NOS. Cell lysis is evident after one hr of incubation.

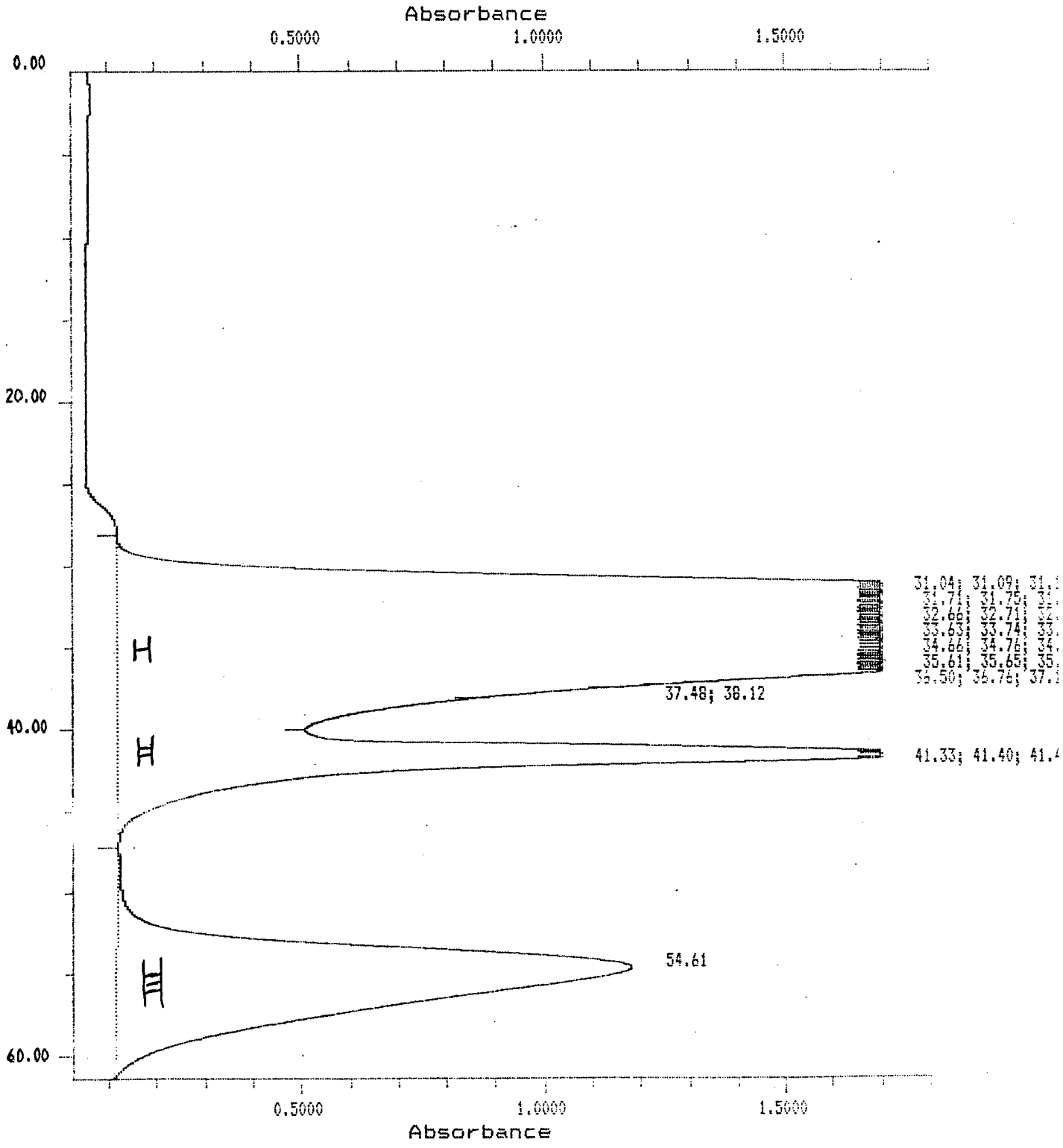
Figure 4B shows zebra fish embryos treated with 25 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 10:1 ratio of Genistein to ANB-NOS. Cell lysis is present here as well.

Figure 4C Zebra fish embryo showing normal development.

EGF-Genistein conjugates prepared using the ANB-NOS crosslinker at a 1:10

Figures 5A - 5D - MTT assays using SQ-20B and SK-BR-3 cell lines. EGF alone was tested as well as various EGF-Gen conjugates (Fig. 5A - EGF-SANPAH-Genistein, Fig. 5B - EGF-SFAD-Genistein), EGF-SANPAH-P97 (Fig.5A), and EGF-24 conjugates (Fig. 5C and 5D). The EGF/24 conjugate was formed by direct linkage of DDE24 to EGF (i.e. no crosslinker was used). EGF/ANBNOS-24 was made by prephotolyzing DDE24 and the ANBNOS crosslinker. NPP in the figures means not prephotolyzed; EGF was modified and subsequently photolyzed for one hr in the presence of Genistein or P97.

Figure 1- Figure 1 shows an example of a size-exclusion HPLC profile of an EGF-Genistein conjugate prepared using a 4:1 ratio of crosslinker to EGF and a prephotolyzed mixture containing a 10:1 molar excess of Genistein to SANPAH. The Beckman System Gold HPLC was equipped with a TSKG3000PW analytical column equilibrated in 100 mM sodium phosphate buffer, pH 6.8, at a flow rate of 0.2 mL/minute. Fractions are labeled I, II, and III.



Data File C:\HPCHEM\1\DATA\LISAR\EGFS001.D Sample Name: EGF/SAN, #

EGF/SAN (1:4), after PD-10 & NAP-10 purification. Reac
tion 4, 5/11/99. 5 mg/ml mix.
A: H20, 0.1% TFA. D: 80% ACN, 20% H2O, 0.1% TFA.
Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;
t=20, 43.5 % D; t=35, 100% D; t=50, 100% D, t=55, 20% D
; t=56, stop. Flow=1.0 mL/min.

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Injection Date : 5/14/99 6:53:11 PM Seq. Line : 2
Sample Name : EGF/SAN, #4 Vial : 3
Acq. Operator : L. Kuehn Inj : 1
Inj Volume : 50 µl

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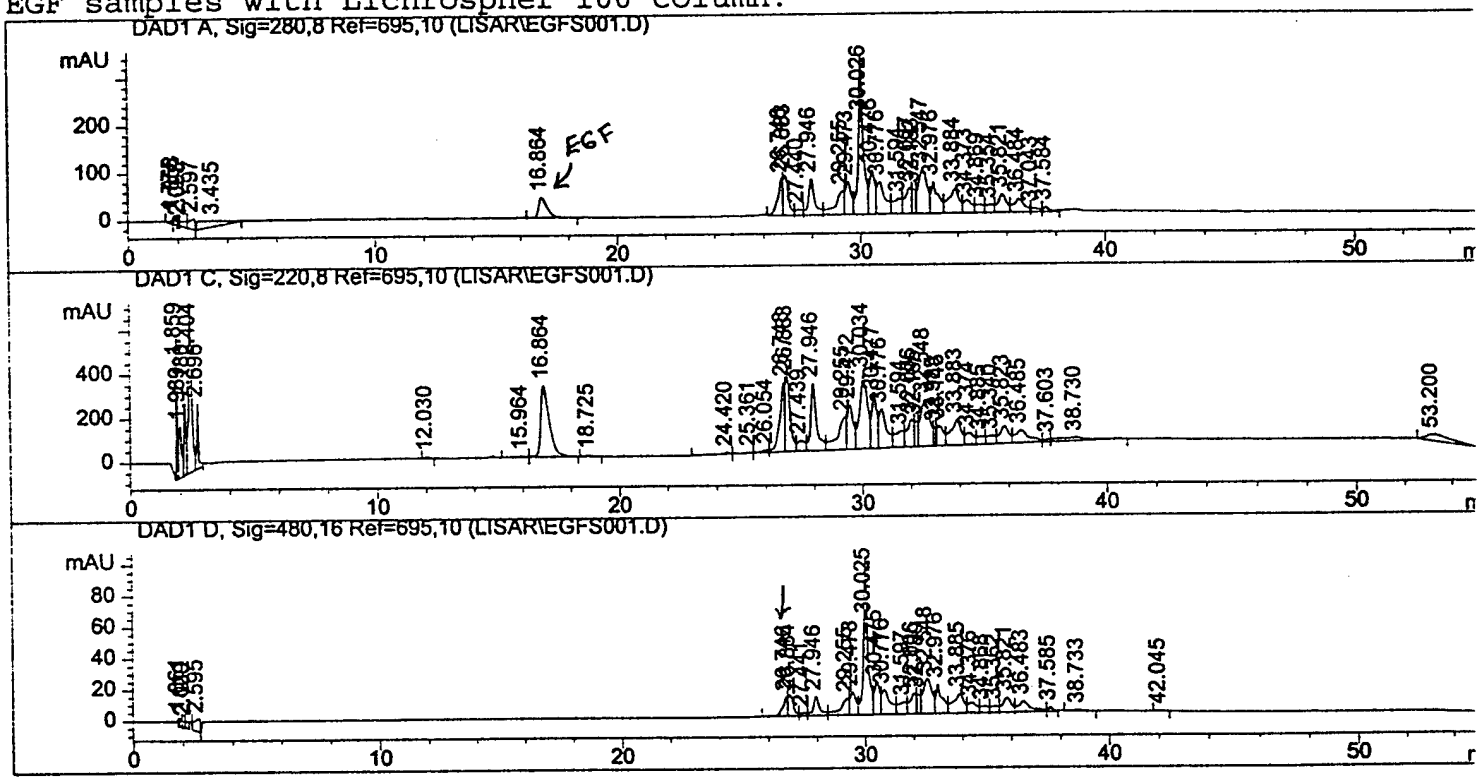

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Print of window 39: UV Apex spectrum of Peak 16.864 of EGFS001.D

UV Apex spectrum of Peak 16.864 of EGFS001.D

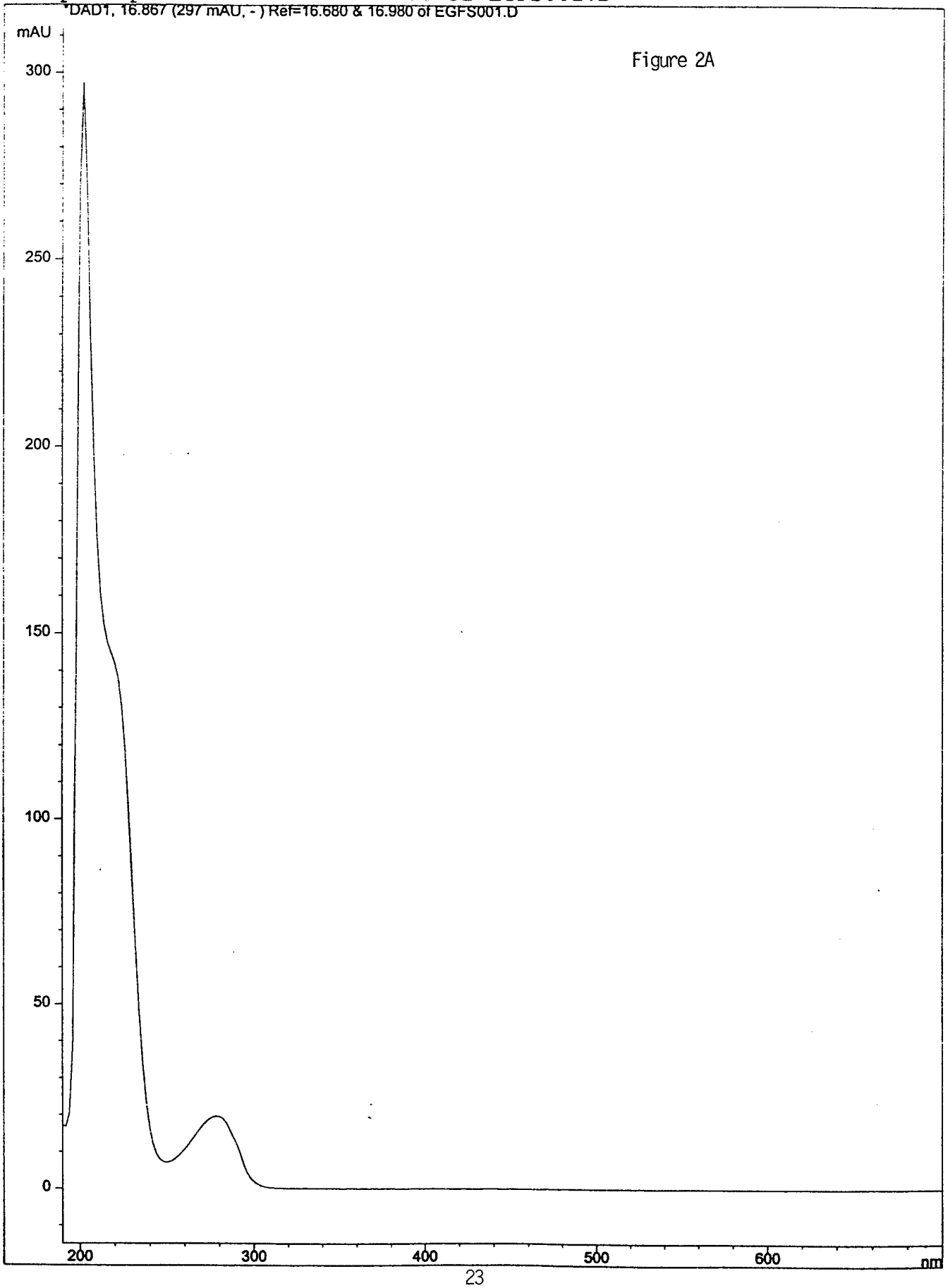

Figure 2A

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UV Apex spectrum of Peak 26.883 of EGFS001.D

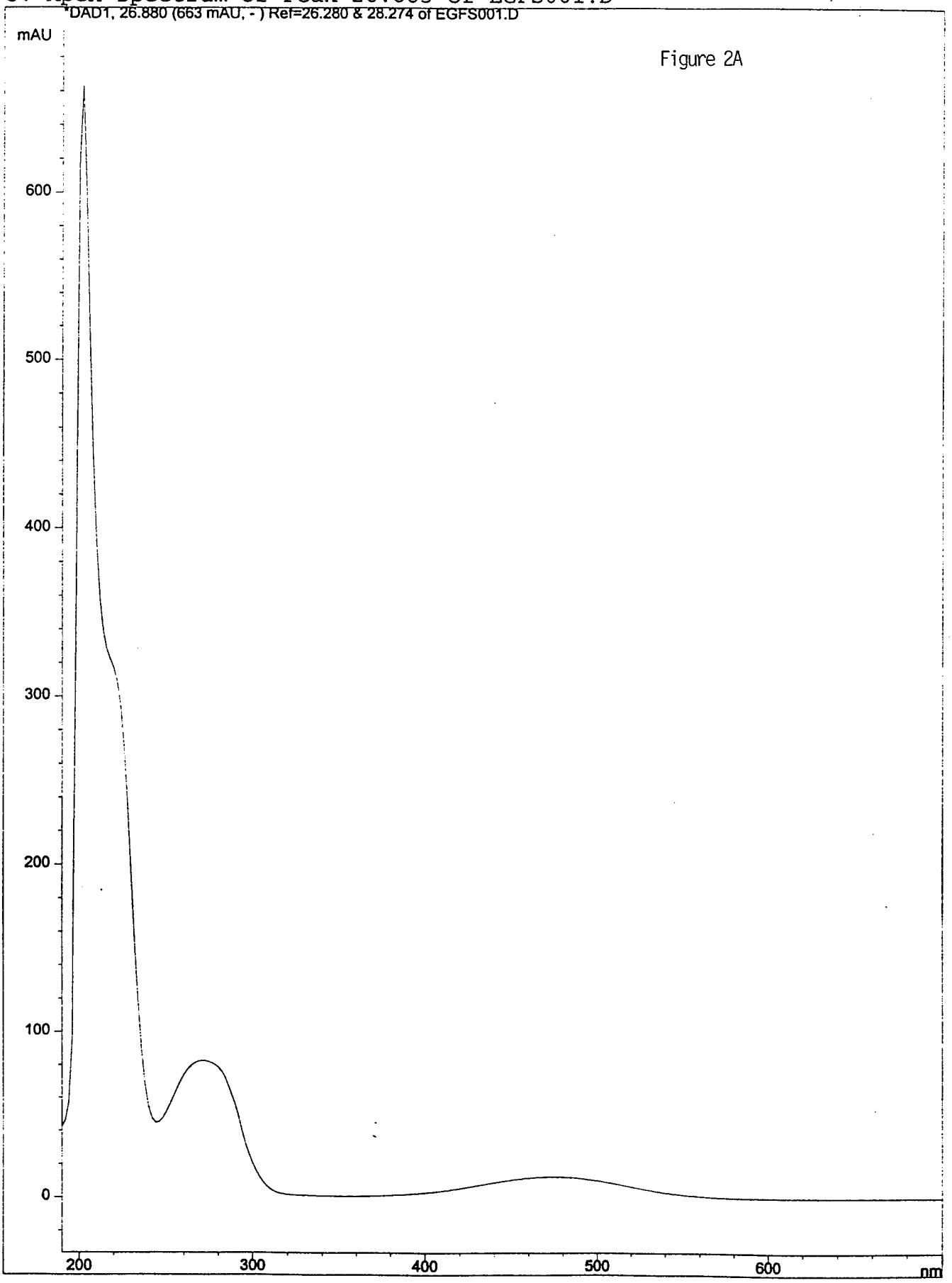


Figure 2A

Print of window 39: UV Apex spectrum of Peak 27.946 of EGFS001.D

UV Apex spectrum of Peak 27.946 of EGFS001.D

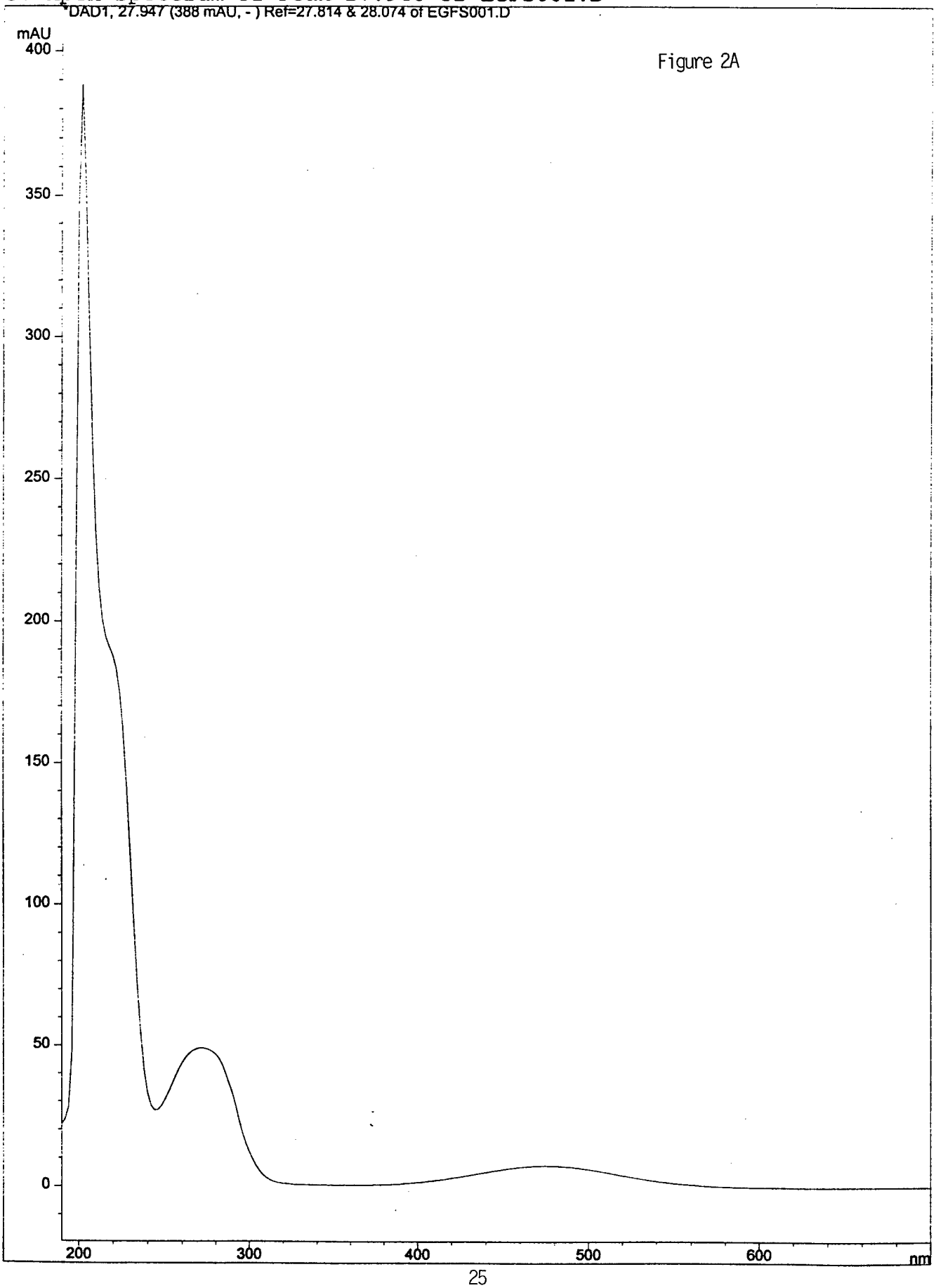


Figure 2A

Print of window 39: UV Apex spectrum of Peak 30.026 of EGFS001.D

UV Apex spectrum of Peak 30.026 of EGFS001.D

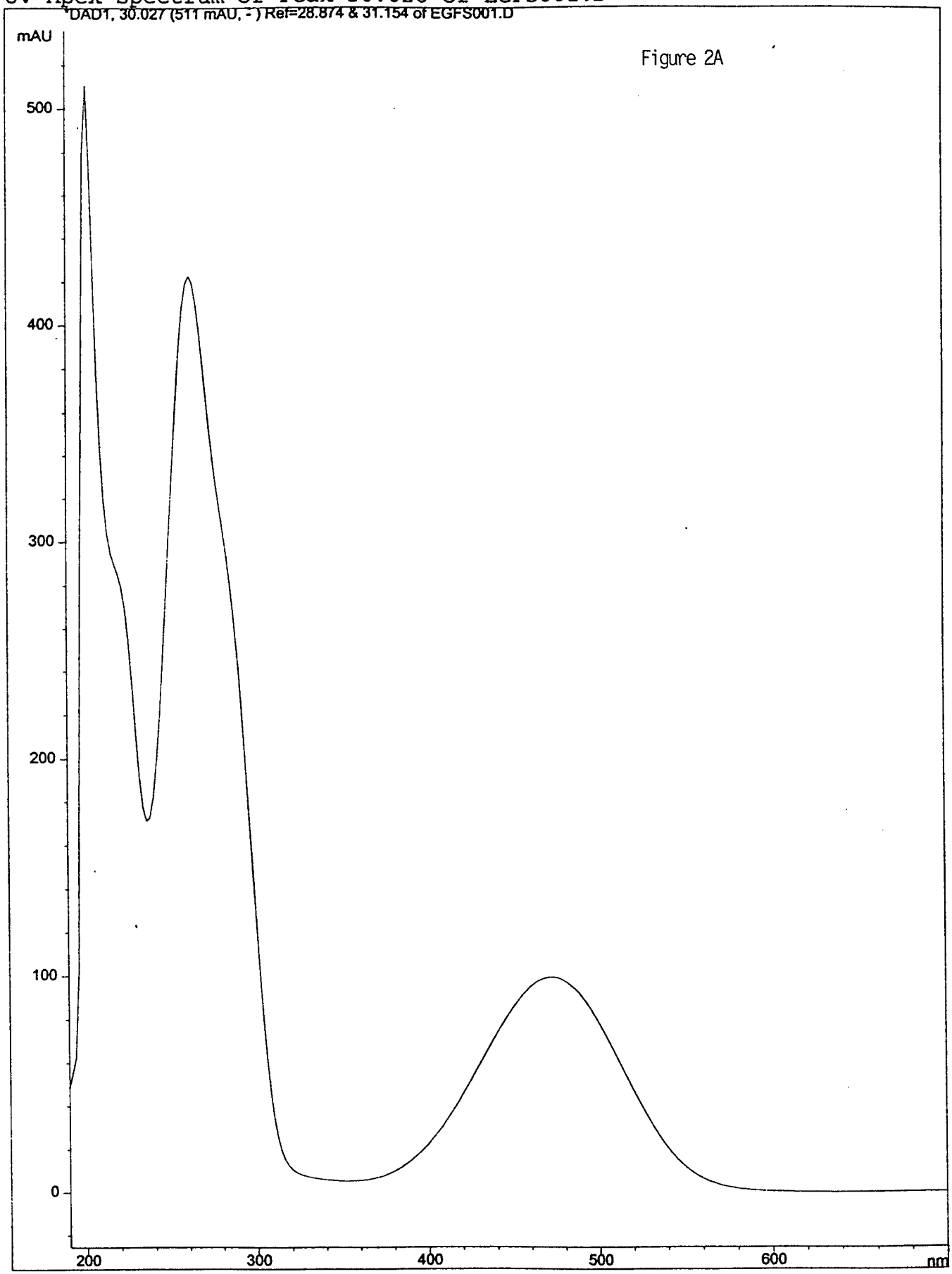


Figure 2A

Print of window 39: UV Apex spectrum of Peak 32.548 of EGFS001.D

UV Apex spectrum of Peak 32.548 of EGFS001.D

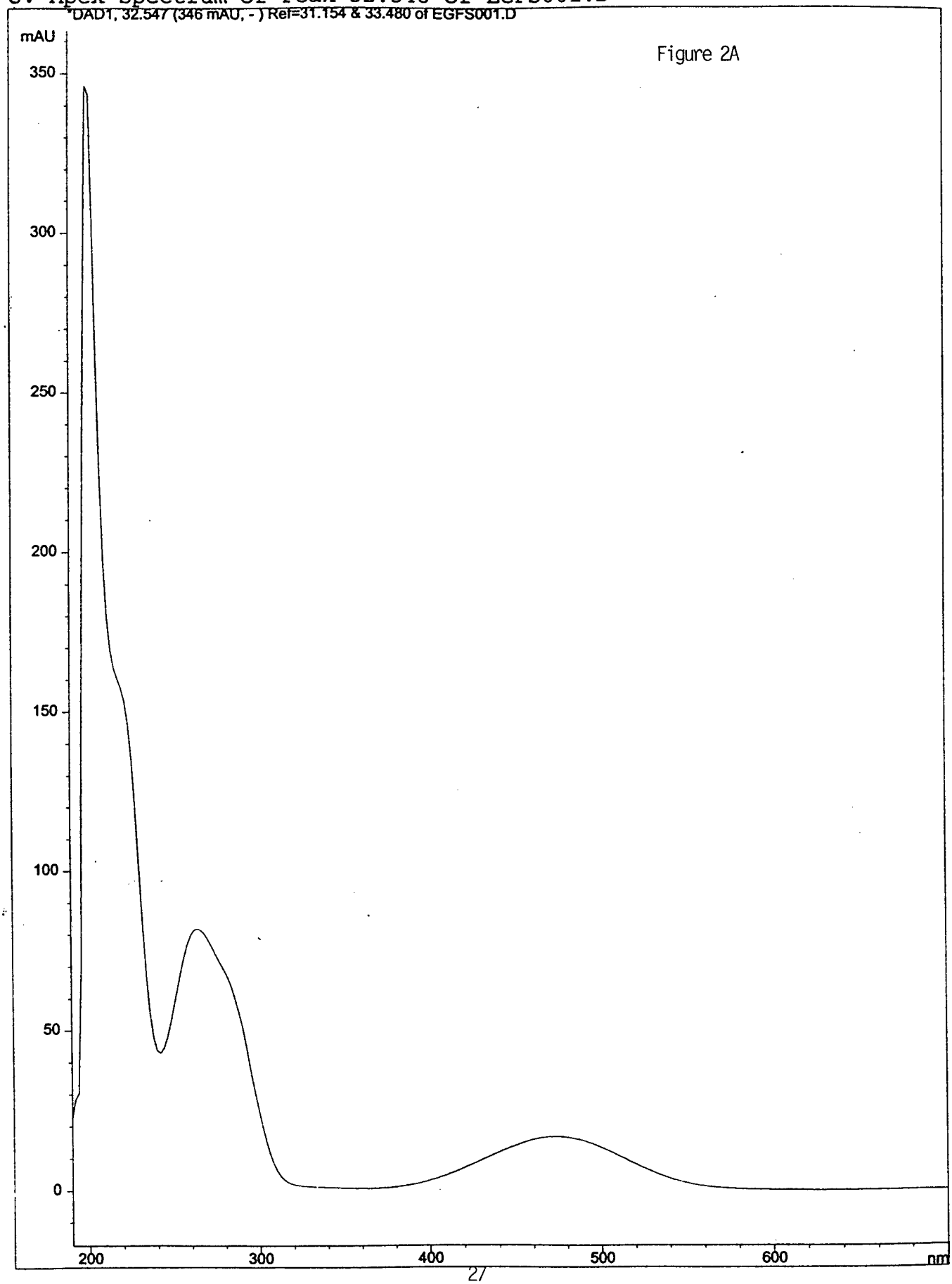


Figure 2A

Data File C:\HPCHEM\1\DATA\LISAR\EGFSG003.D Sample Name: EGF/SAN/Gen, #

EGF/SAN-Gen (1:4)(1:10), not pp. Reaction 4, 5/12/99. 1hLw uv
A: H20, 0.1% TFA. D: 80% ACN, 20% H2O, 0.1% TFA.
Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;
t=20, 43.5 % D; t=35, 100% D; t=50, 100% D, t=55, 20% D
; t=56, stop. Flow=1.0 mL/min.

==========

Injection Date	: 5/14/99 8:00:34 PM	Seq. Line	: 3
Sample Name	: EGF/SAN/Gen, #4	Vial	: 4
Acq. Operator	: L. Kuehn	Inj	: 1
		Inj Volume	: 50 µl

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M
Last changed : 5/14/99 4:15:43 PM by L. Kuehn
EGF samples with Lichrospher 100 column.

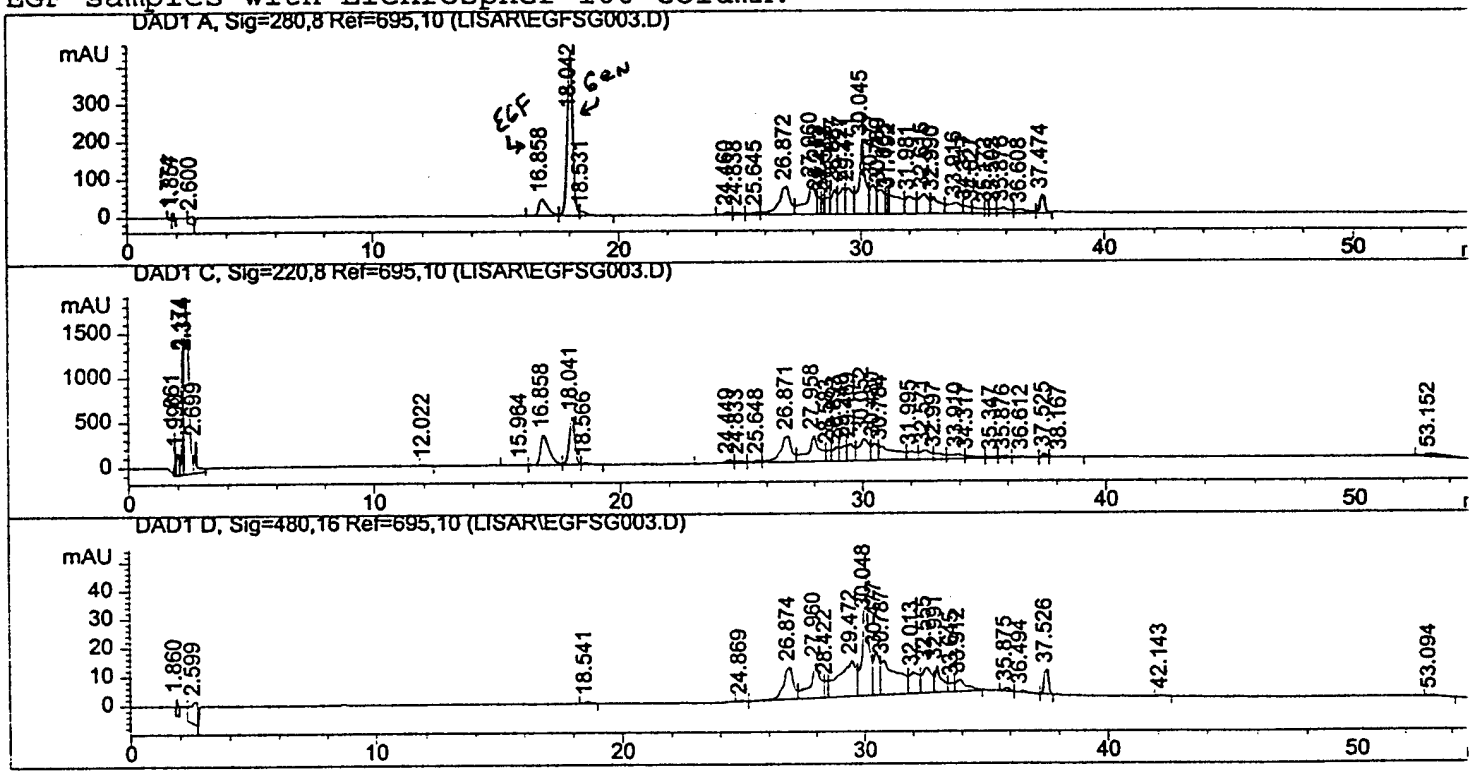


Figure 2B - Figure 2B shows a reverse-phase HPLC pattern of EGF-Genistein made by photolyzing the EGF-SANPAH in the presence of a 10-fold molar excess of Genistein. In addition to the unmodified EGF(retention time of 16.858 min), a peak of unreacted Genistein is also present at 18.042 min. UV spectra are included for representative peaks; the peak eluting at 37.474 min. appears to have characteristics of EGF, SANPAH, and Genistein.

UV Apex spectrum of Peak 18.042 of EGFSG003.D

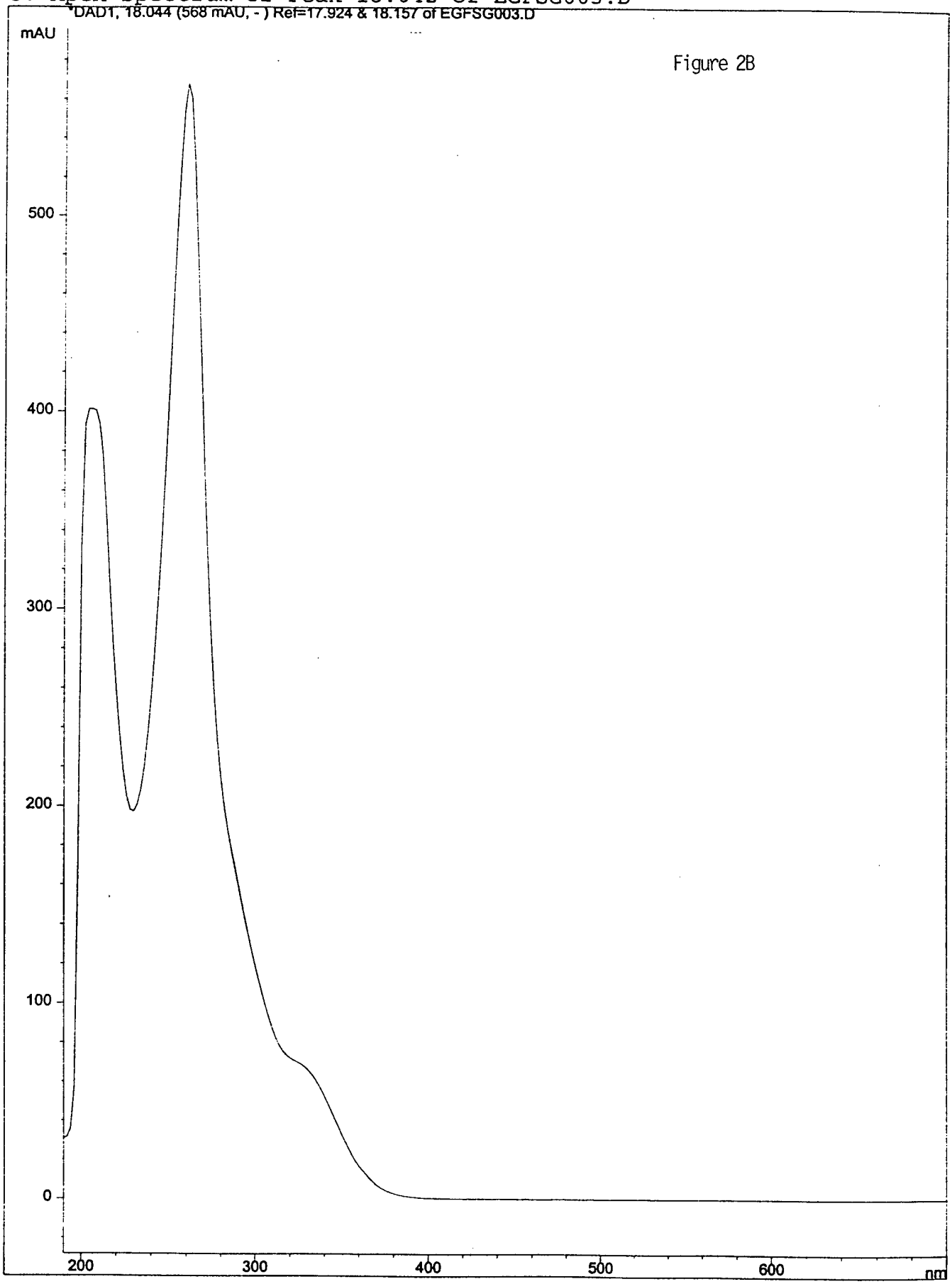


Figure 2B

Print of window 39: UV Apex spectrum of Peak 26.872 of EGFSG003.D

UV Apex spectrum of Peak 26.872 of EGFSG003.D

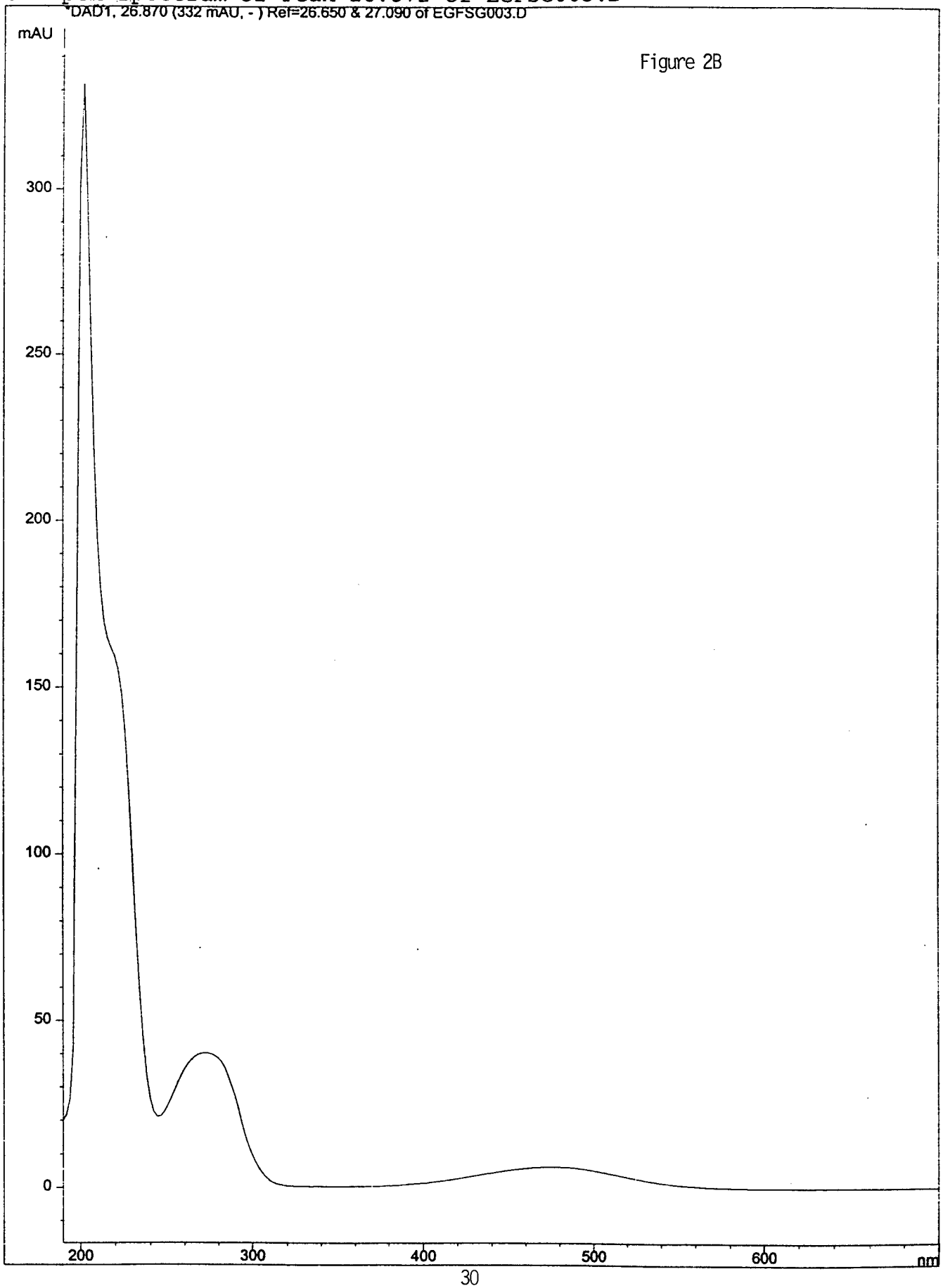


Figure 2B

Instrument 1 5/19/99 5:09:48 PM Lisa Kuehn

Print of window 39: UV Apex spectrum of Peak 27.958 of EGFSG003.D

UV Apex spectrum of Peak 27.958 of EGFSG003.D

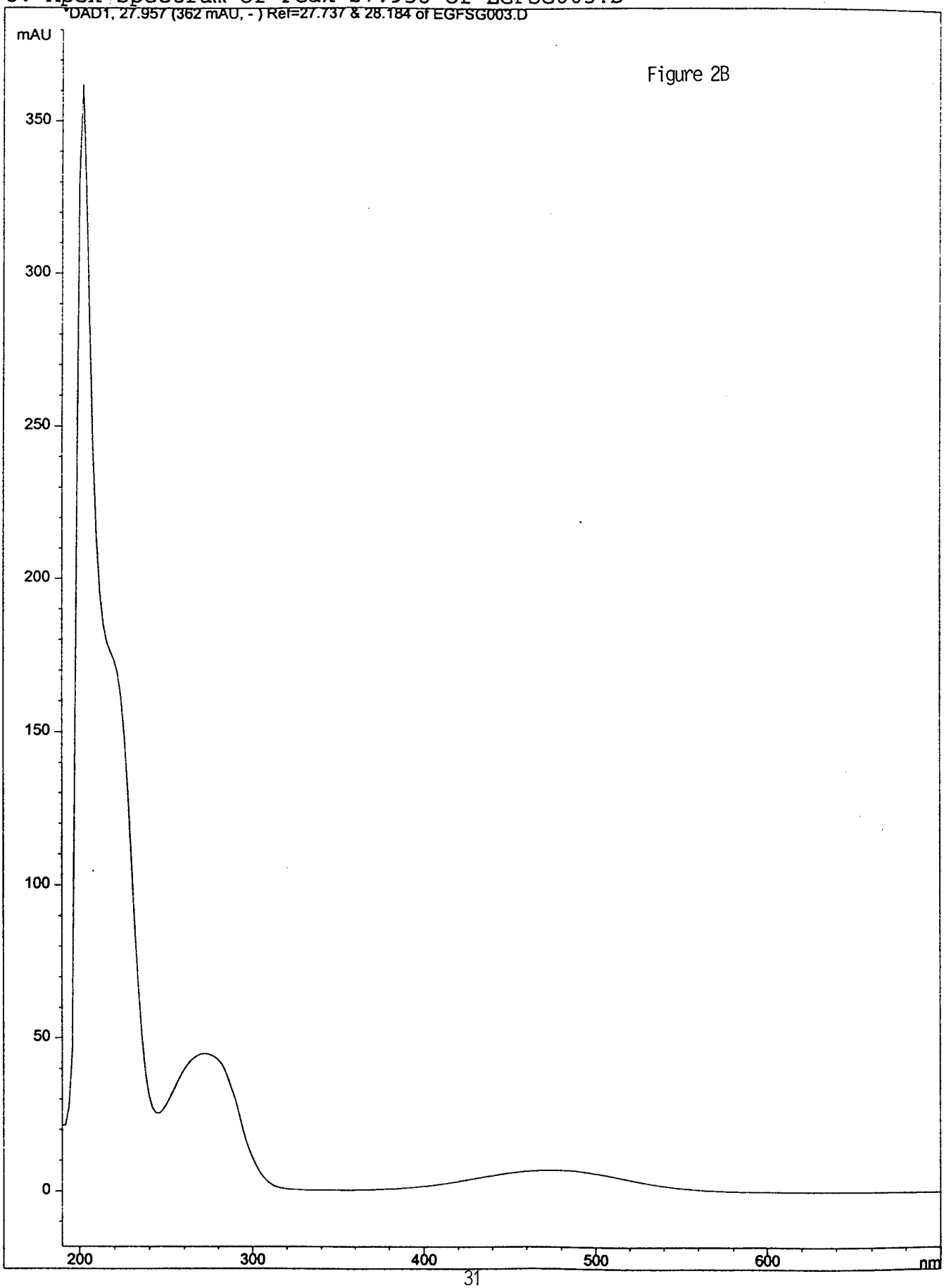


Figure 2B

Print of window 39: UV Apex spectrum of Peak 30.045 of EGFSG003.D

UV Apex spectrum of Peak 30.045 of EGFSG003.D

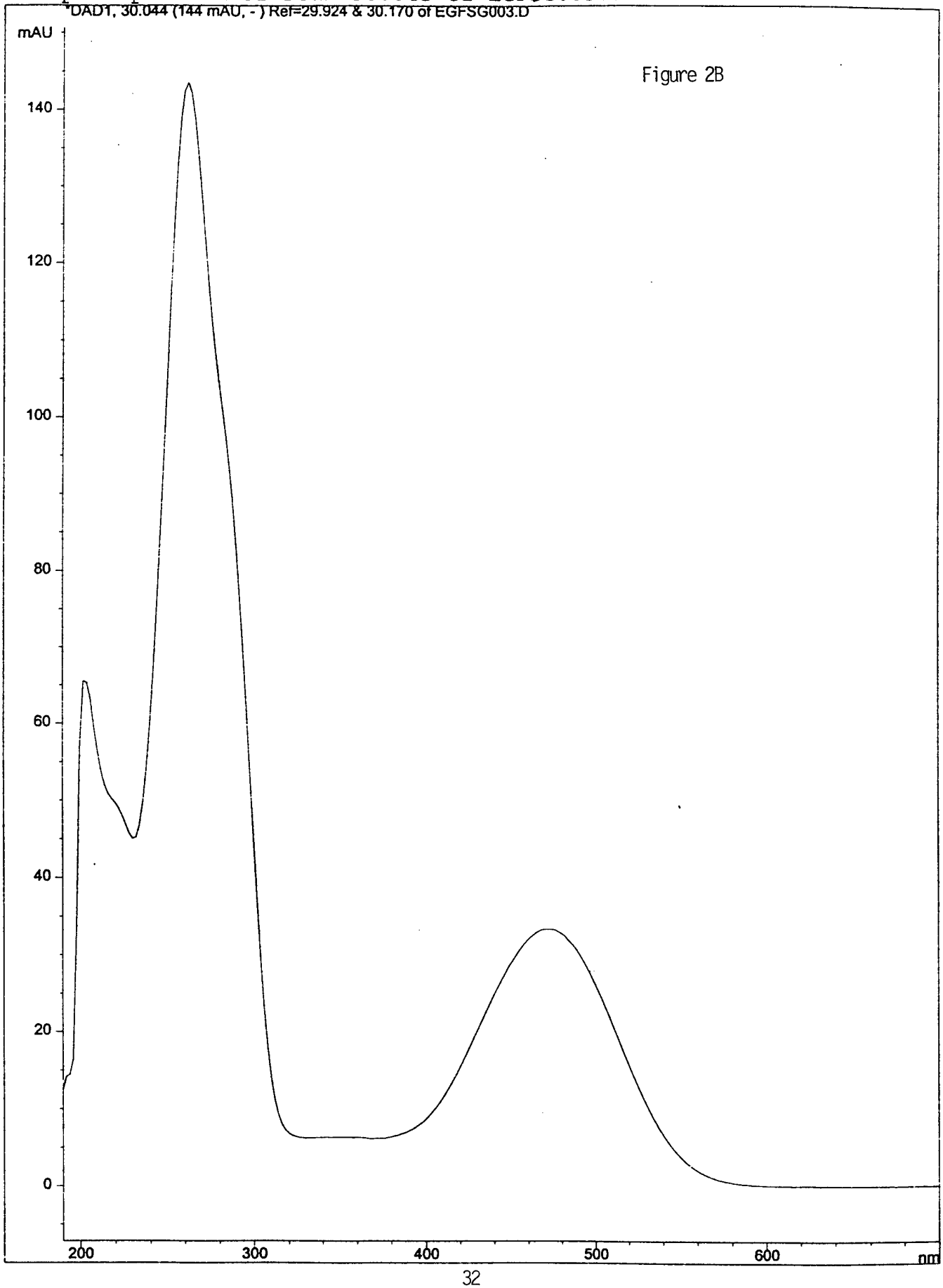


Figure 2B

Print of window 39: UV Apex spectrum of Peak 37.474 of EGFSG003.D

UV Apex spectrum of Peak 37.474 of EGFSG003.D

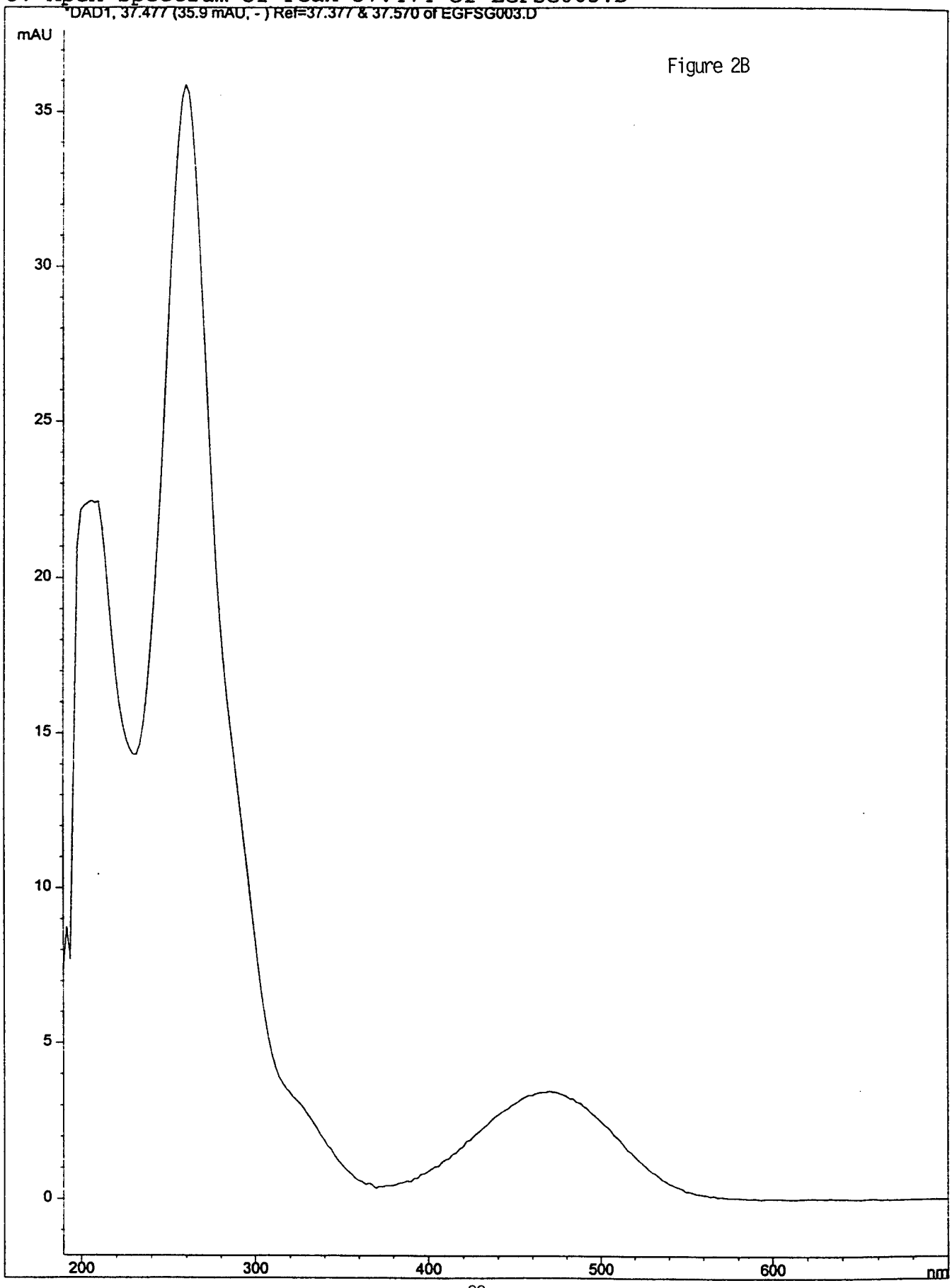


Figure 2B

Instrument 1 5/19/99 5:10:41 PM Lisa Kuehn

Data File C:\HPCHEM\1\DATA\LISAR\EGFSG007.D Sample Name: EGF/SAN-Gen p

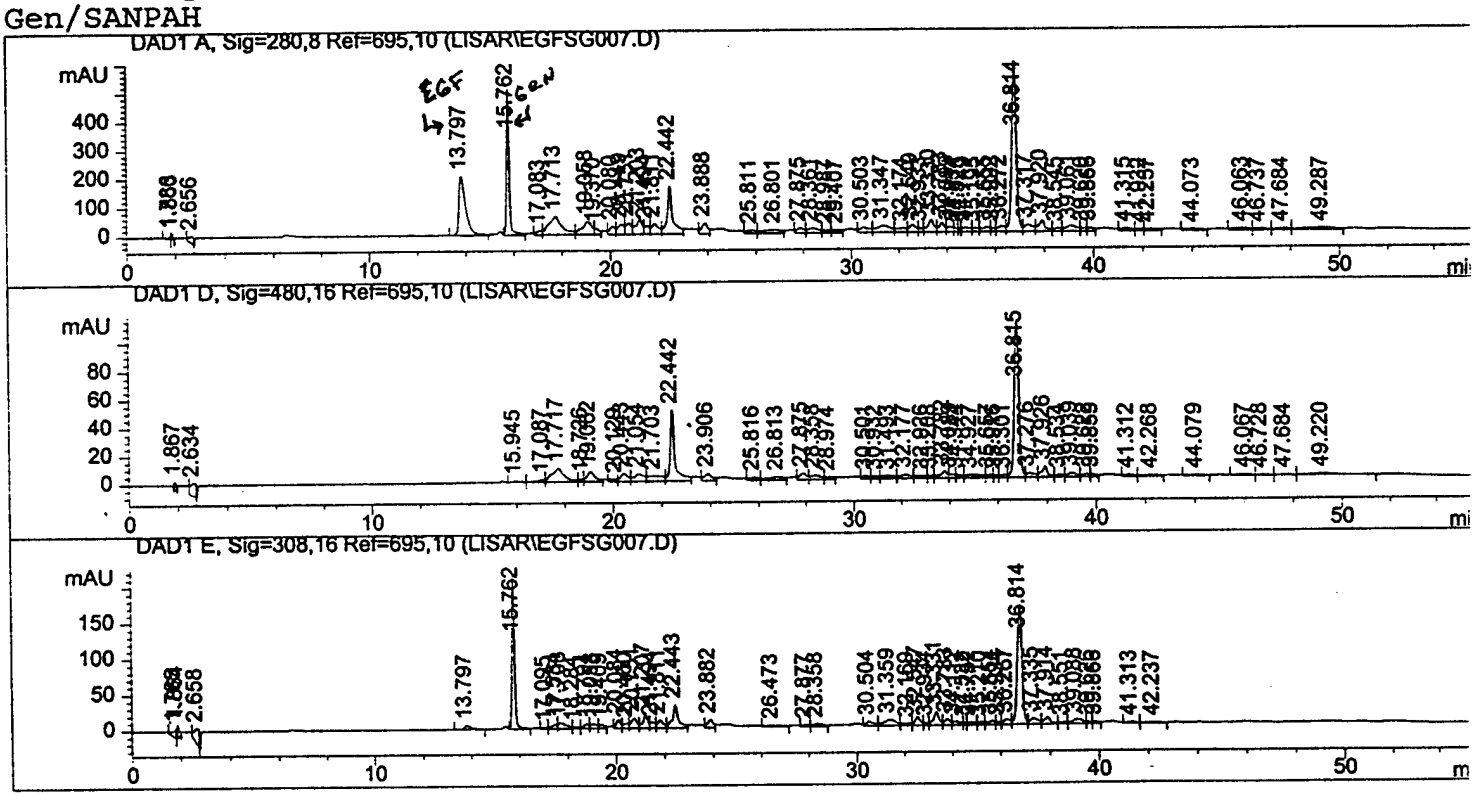
EGF/SAN-Gen 1:4, 1:10. 16.5 hr LWUV. 5 mg/mL origina
l. pre-photolyzed
5/19/99. Parent compound
A: H2O, 0.1%TFA, 0.1%TEA. D: 80% ACN, 20% H2O, 0.1%
TFA.
Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;
t=20, 43.5% D; t=35, 100% D; t=50, 100% D; t=55, 20% D;
t=56, stop. Flow = 1 mL/min.

==========

Injection Date : 5/20/99 6:30:54 AM
Sample Name : EGF/SAN-Gen p
Acq. Operator : Lisa Kuehn

Seq. Line : 10
Vial : 13
Inj : 1
Inj Volume : 50 µl

Acq. Method : C:\HPCHEM\1\METHODS\LISAEGF.M
Last changed : 5/19/99 4:21:34 PM by Lisa Kuehn
Analysis Method : C:\HPCHEM\1\METHODS\LISAGEN3.M
Last changed : 5/11/99 2:46:56 PM by L. Kuehn
Gen/SANPAH



==========

Figure 2C - Figure 2C shows a reverse-phase HPLC pattern of EGF-Genistein made by prephotolyzing the SANPAH/Genistein mixture prior to adding the EGF. The pattern shows peaks characteristic of unmodified EGF and Genistein, as well as of a possible EGF-Genistein conjugate.

Print of window 39: UV Apex spectrum of Peak 13.797 of EGFSG007.D

UV Apex spectrum of Peak 13.797 of EGFSG007.D

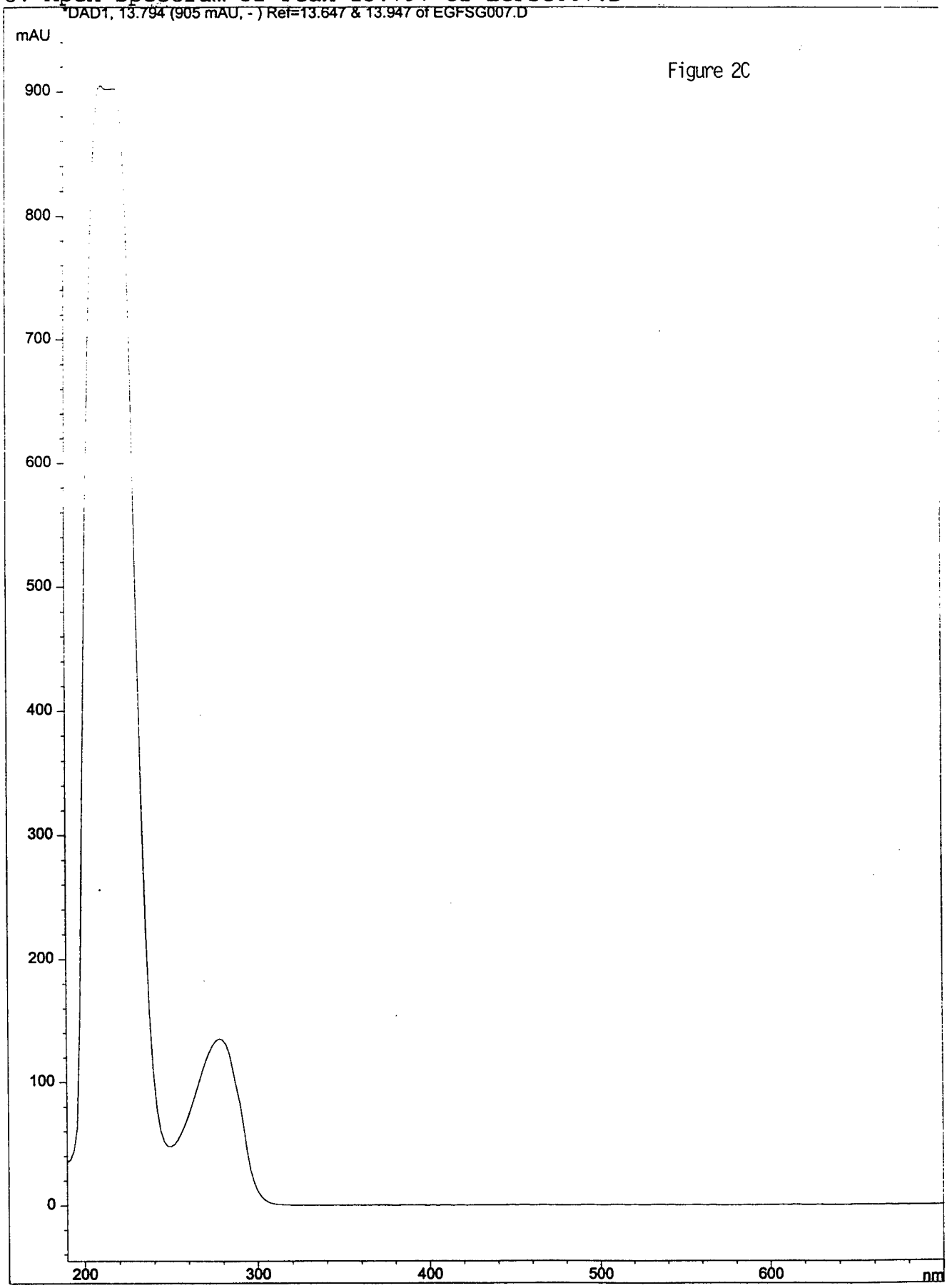


Figure 2C

Instrument 1 5/21/99 2:39:39 PM Lisa Kuehn

Print of window 39: UV Apex spectrum of Peak 15.762 of EGFSG007.D

UV Apex spectrum of Peak 15.762 of EGFSG007.D

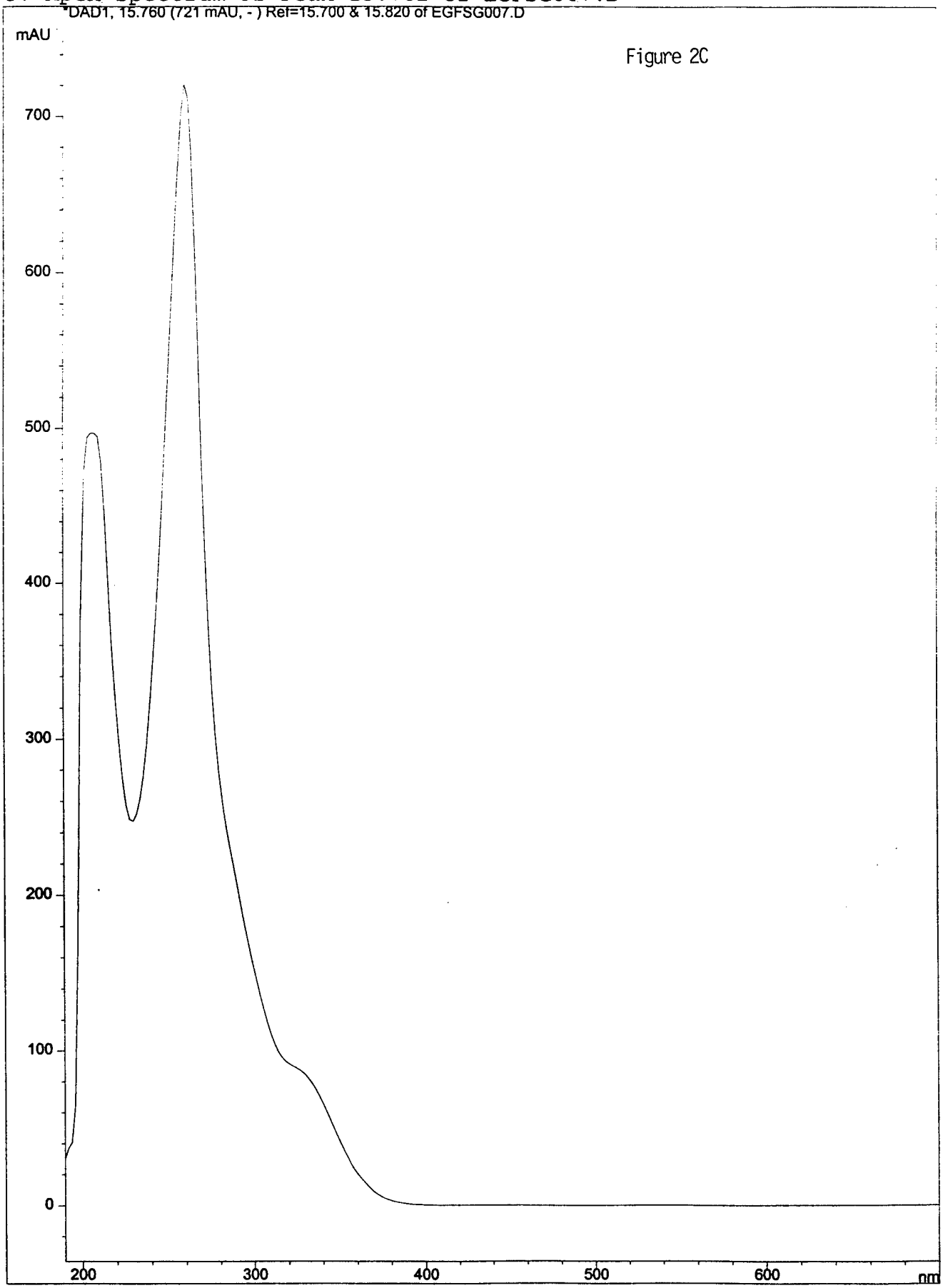


Figure 2C

Instrument 1 5/21/99 2:31:32 PM Lisa Kuehn

Print of window 39: UV Apex spectrum of Peak 17.713 of EGFSG007.D

UV Apex spectrum of Peak 17.713 of EGFSG007.D

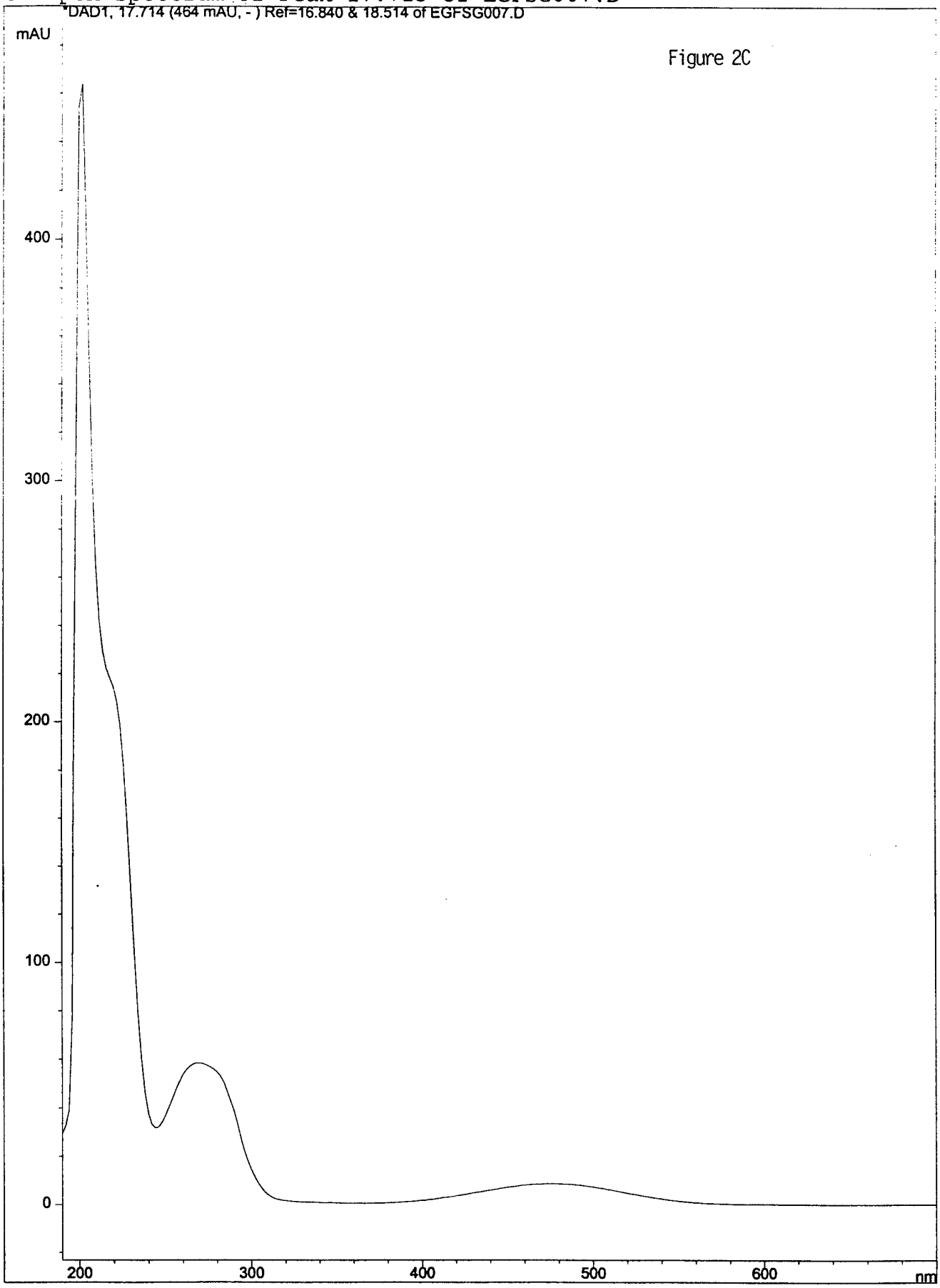


Figure 2C

Instrument 1 5/21/99 2:32:30 PM Lisa Kuehn

Print of window 39: UV Apex spectrum of Peak 22.442 of EGFSG007.D

UV Apex spectrum of Peak 22.442 of EGFSG007.D

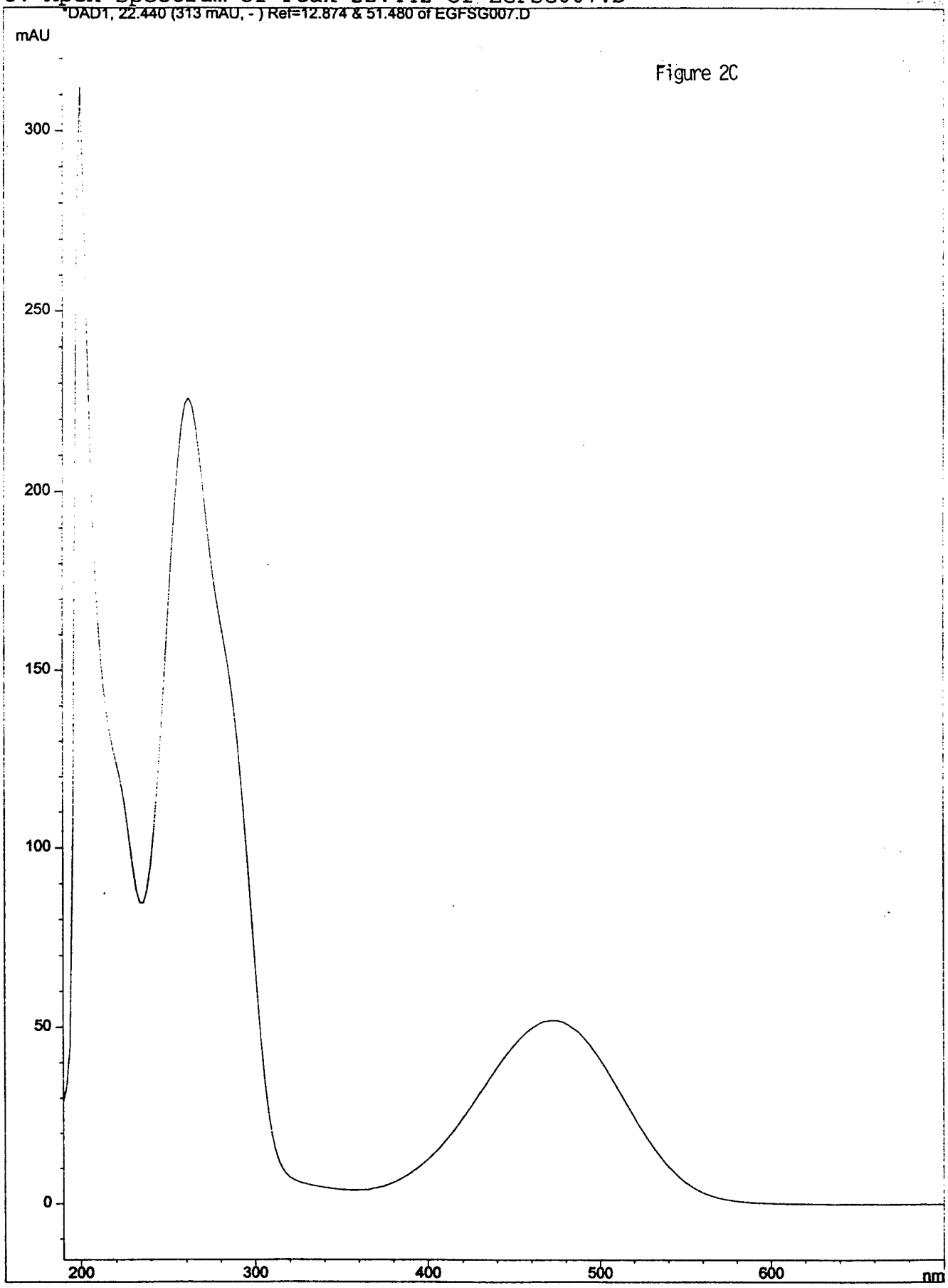


Figure 2C

Instrument 1 5/21/99 2:33:23 PM Lisa Kuehn

Print of window 39: UV Apex spectrum of Peak 36.814 of EGFSG007.D

UV Apex spectrum of Peak 36.814 of EGFSG007.D

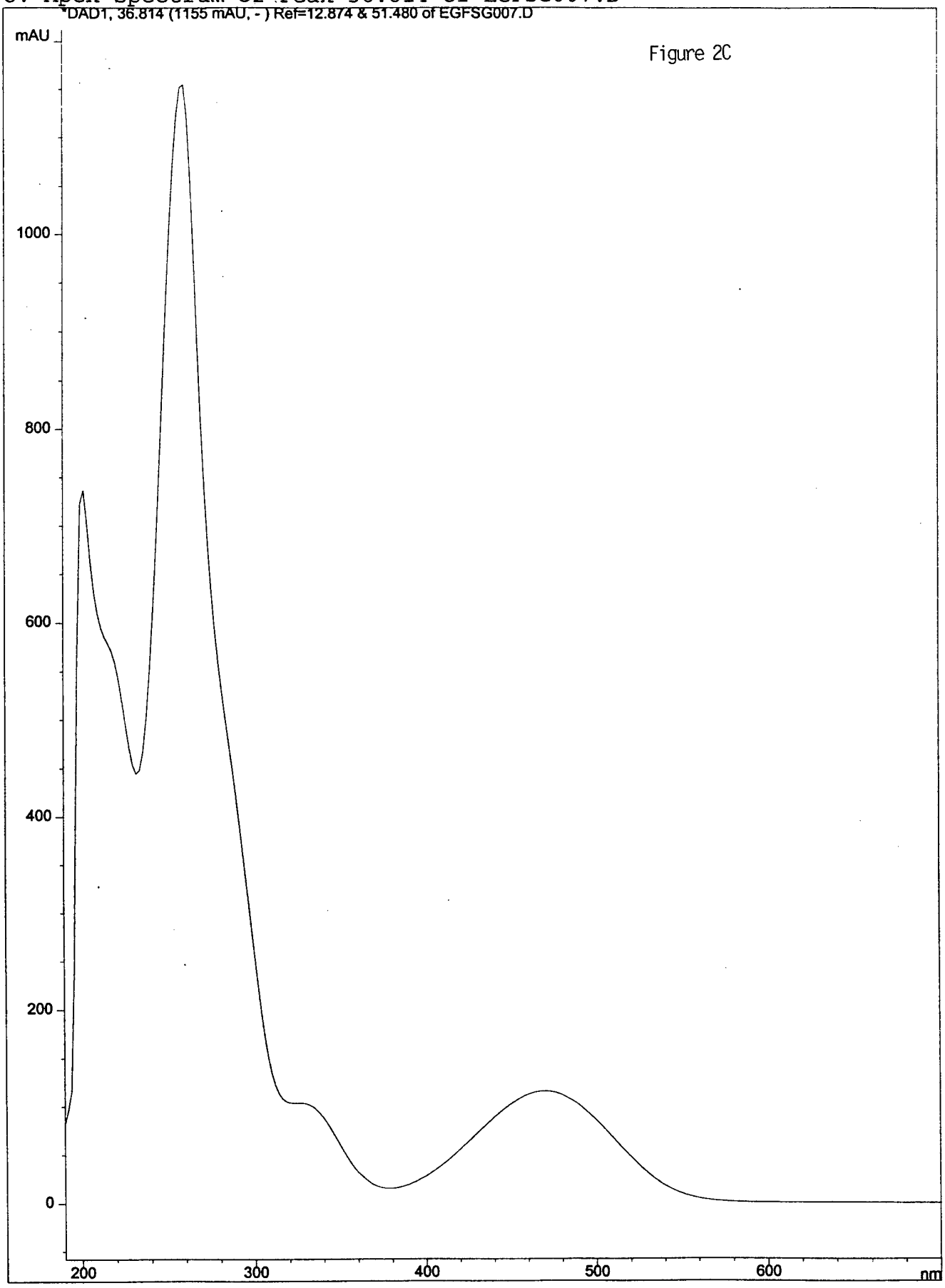


Figure 2C

Instrument 1 5/21/99 2:33:35 PM Lisa Kuehn

Data File C:\HPCHEM\1\DATA\LISAR\EGFSG006.D Sample Name: EGF/SAN-Gen III

EGF/SAN-Gen 1:4, 1:10. 16.5 hr LWUV. 5 mg/mL origina
l.
5/18/99. Analytical III. pre-photolyzed, HPLC III
A: H2O, 0.1%TFA, 0.1%TEA. D: 80% ACN, 20% H2O, 0.1%
TFA.
Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;
t=20, 43.5% D; t=35, 100% D; t=50, 100% D; t=55, 20% D;
t=56, stop. Flow = 1 mL/min.

==========

Injection Date	: 5/19/99 8:49:38 AM	Seq. Line	: 13
Sample Name	: EGF/SAN-Gen III	Vial	: 33
Acq. Operator	: Lisa Kuehn	Inj	: 1
		Inj Volume	: 50 µl
Acq. Method	: C:\HPCHEM\1\METHODS\LISAEGF.M		
Last changed	: 5/12/99 9:27:49 AM by Lisa Kuehn		
Analysis Method	: C:\HPCHEM\1\METHODS\LISAGEN3.M		
Last changed	: 5/11/99 2:46:56 PM by L. Kuehn		

Gen/SANPAH

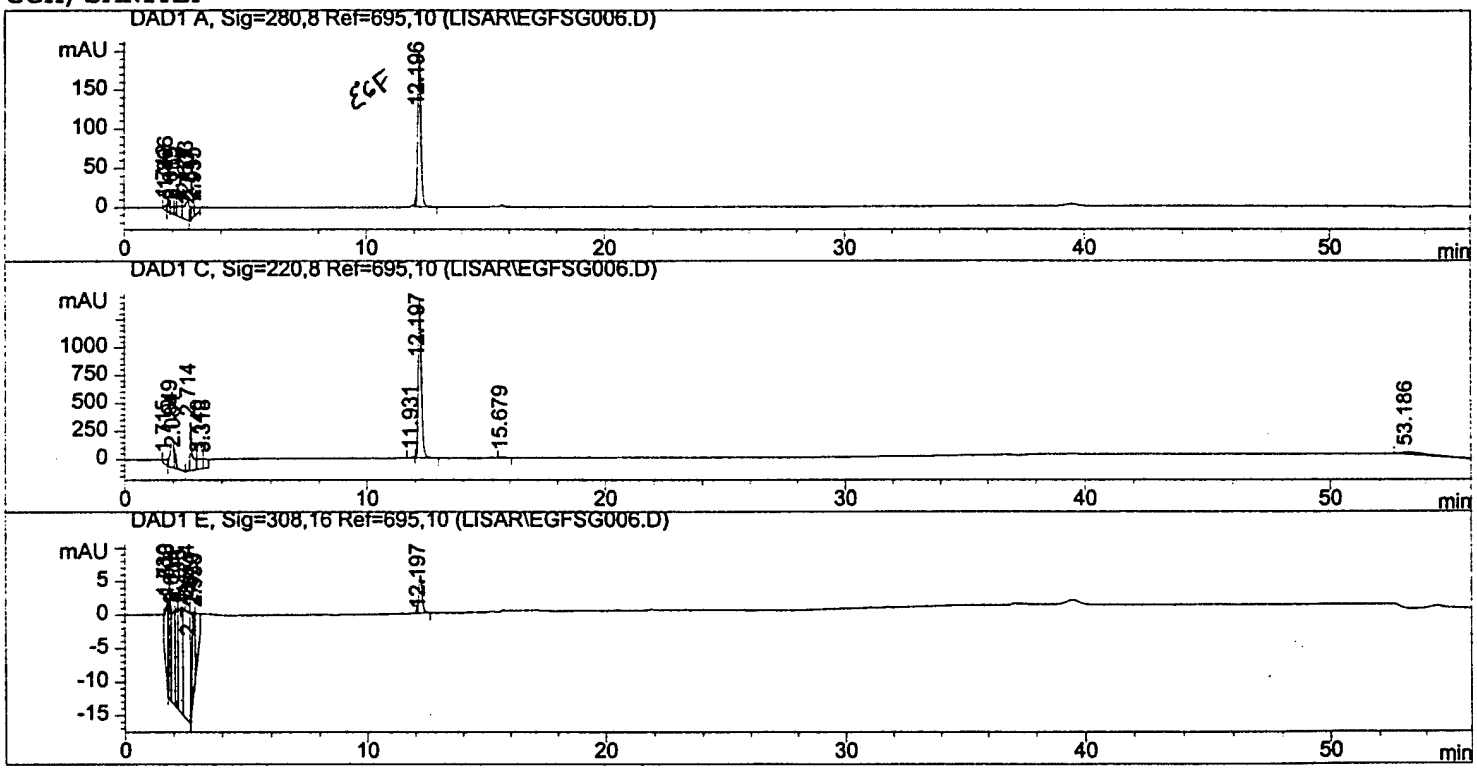


Figure 2D - Figure 2D is a reverse-phase HPLC trace of fraction III shown in **Figure 1** . The peak with a retention time of 12.196 min. and a UV spectrum characteristic of unmodified EGF verifies that the size-exclusion chromatography is able to remove a significant amount of the free EGF remaining in the conjugation mixture.

Print of window 39: UV Apex spectrum of Peak 12.196 of EGFSG006.D

UV Apex spectrum of Peak 12.196 of EGFSG006.D

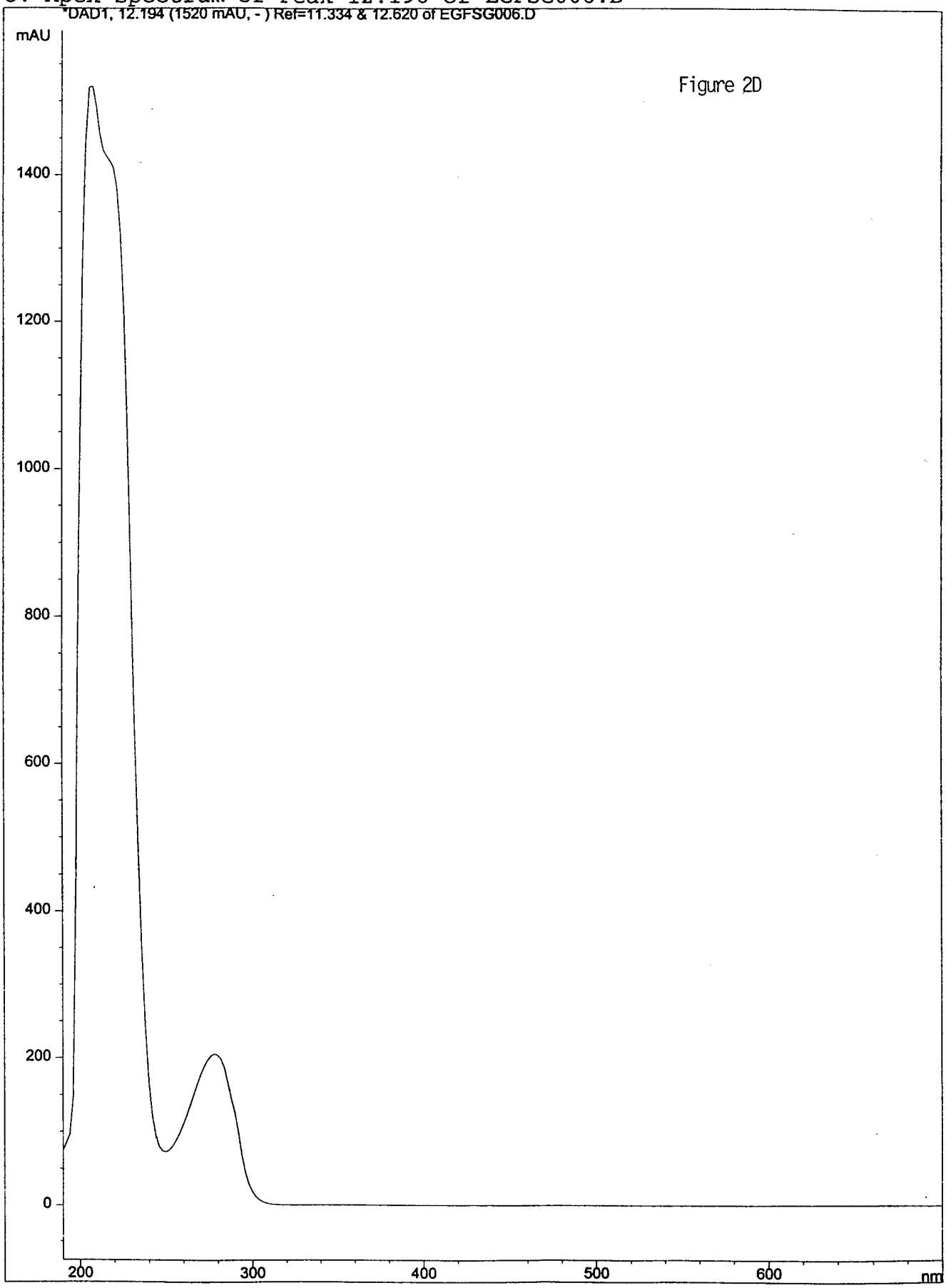
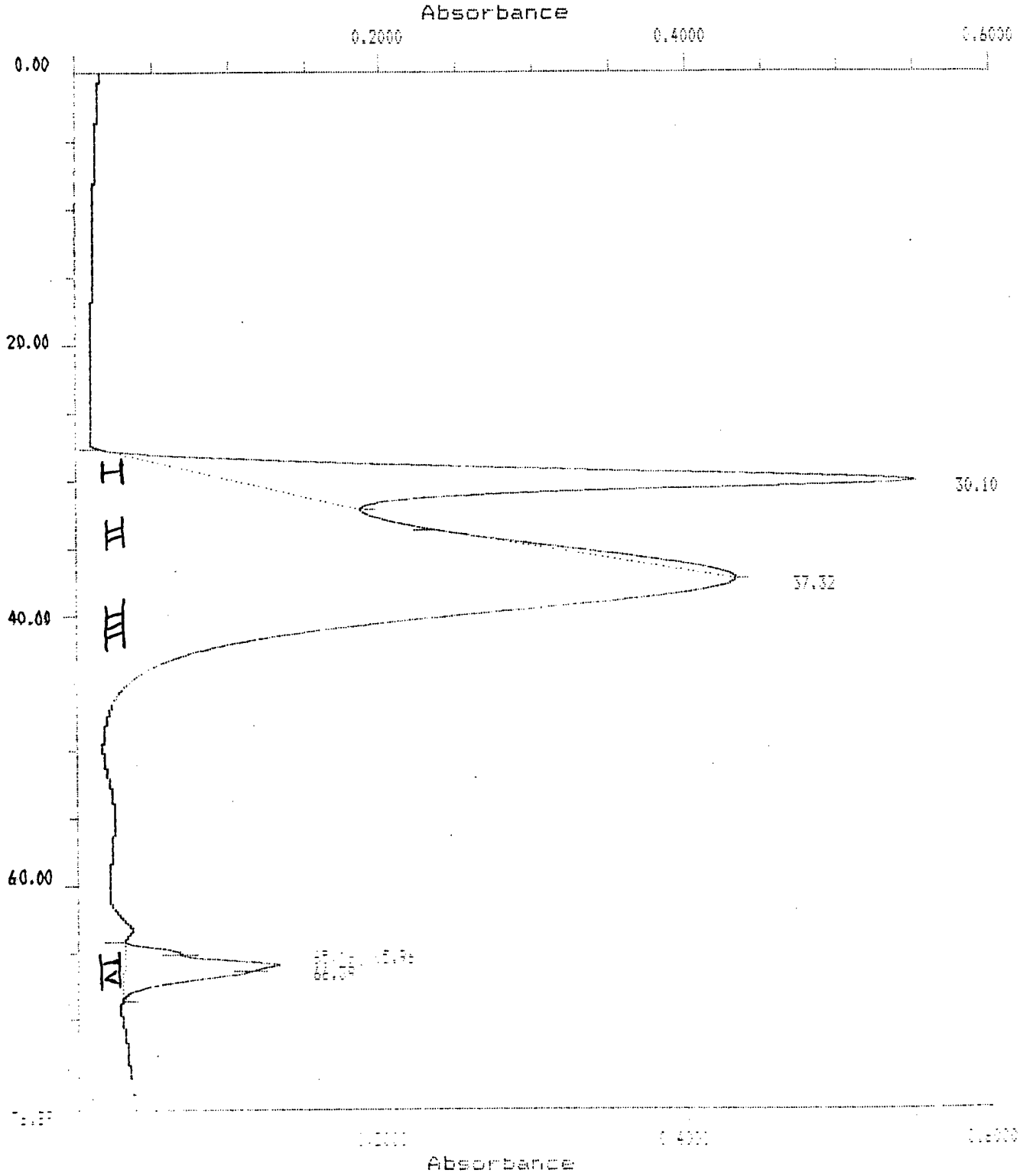


Figure 2D

Instrument 1 5/19/99 4:24:13 PM Lisa Kuehn

Figure 3A shows a preparative size-exclusion purification of an EGF-Genistein conjugate prepared using the prephotolyzed SANPAH/Genistein mixture. Fractions were isolated and tested against the BT-20 breast cancer cell line using the MTT assay(**Figure 3B**). In the MTT assay, the greater the response(y-axis), the greater the cell viability.



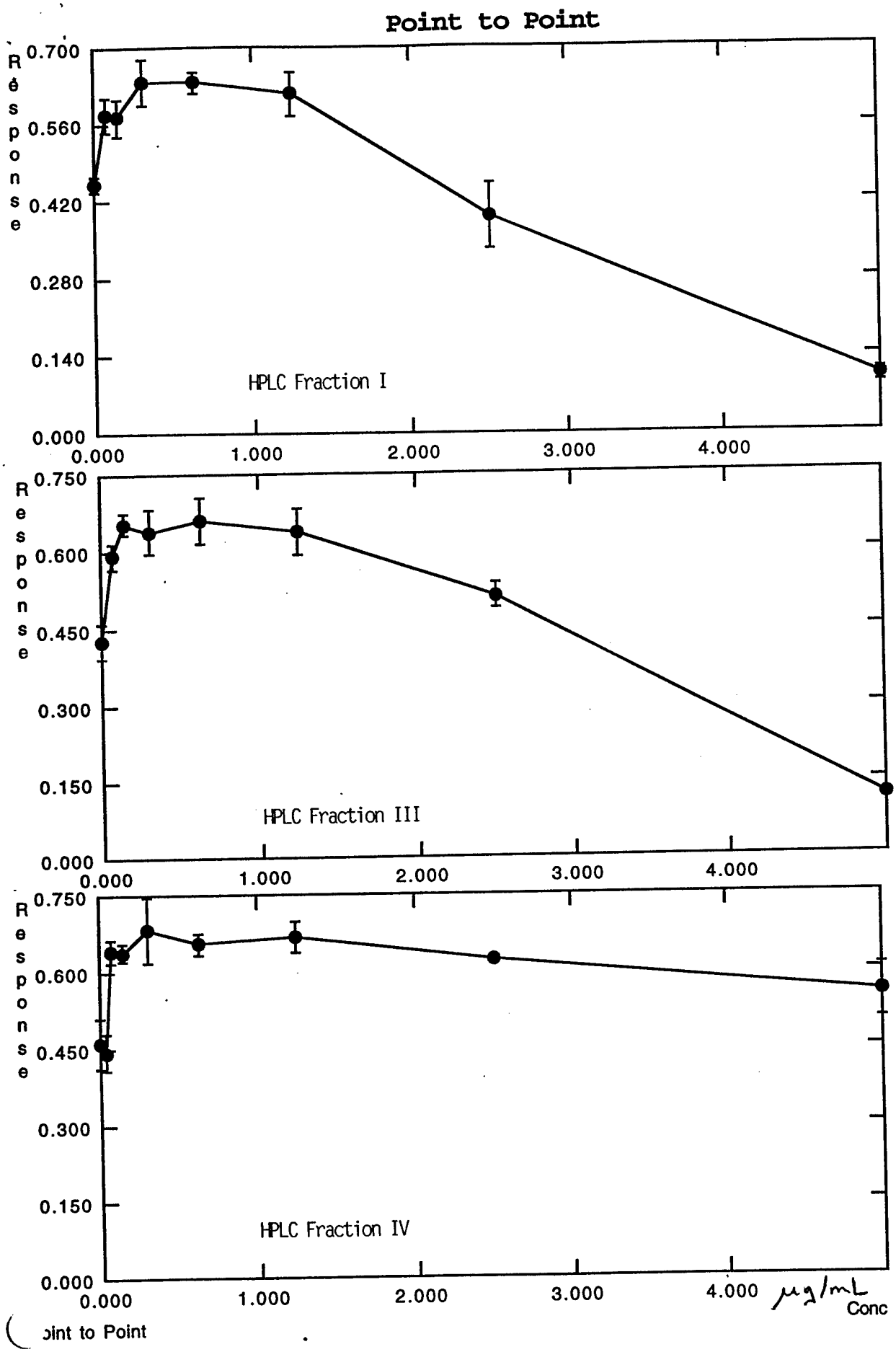


Figure 3B - MTT Assay of EGF-Genistein HPLC Fractions Incubated with BT-20 Cells

Figure 4A shows a zebra fish embryo treated with 50 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 5:1 ratio of Genistein to ANB-NOS. Cell lysis is evident after one hr of incubation.

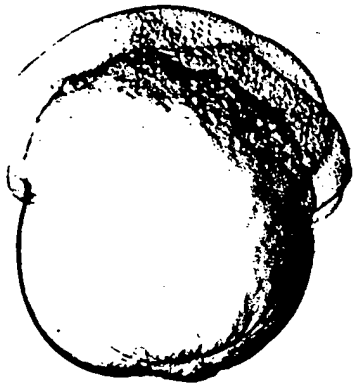
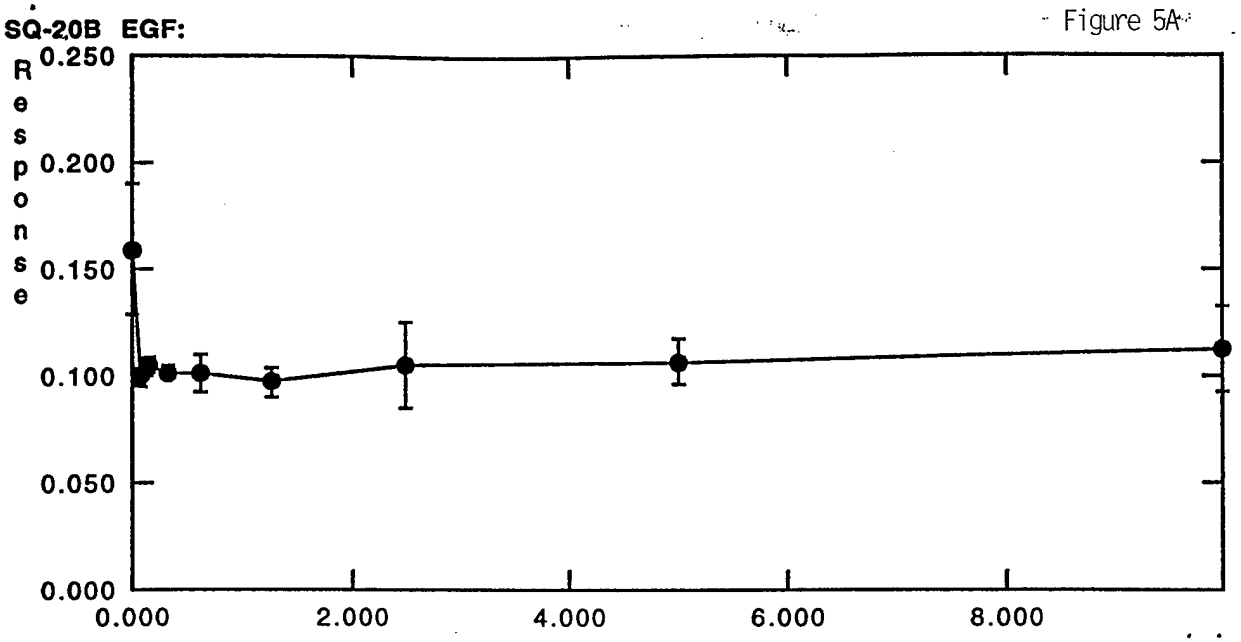
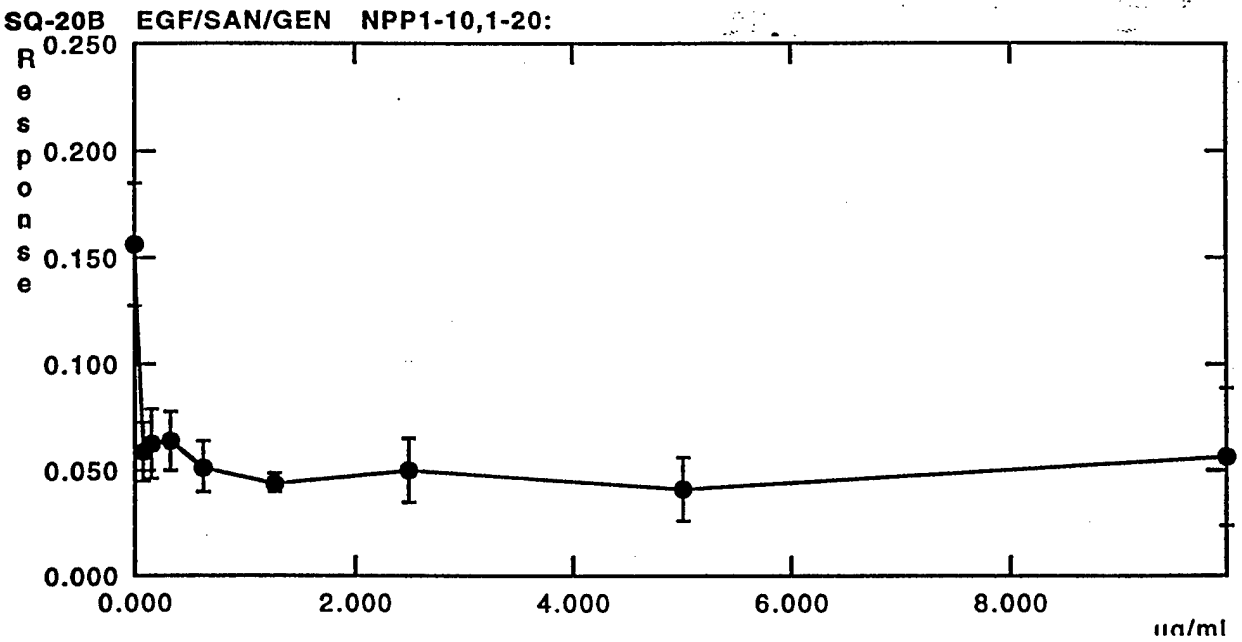


Figure 4B shows zebra fish embryos treated with 25 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 10:1 ratio of Genistein to ANB-NOS. Cell lysis is present here as well.

Figure 4C Zebra fish embryo showing normal development.

Figure 5A


SQ-20B EGF:
Response
0.250
0.200
0.150
0.100
0.050
0.000
0.000
2.000
4.000
6.000
8.000


SQ-20B EGF/SAN/GEN NPP1-10,1-20:
Response
0.250
0.200
0.150
0.100
0.050
0.000
0.000
2.000
4.000
6.000
8.000
ug/ml

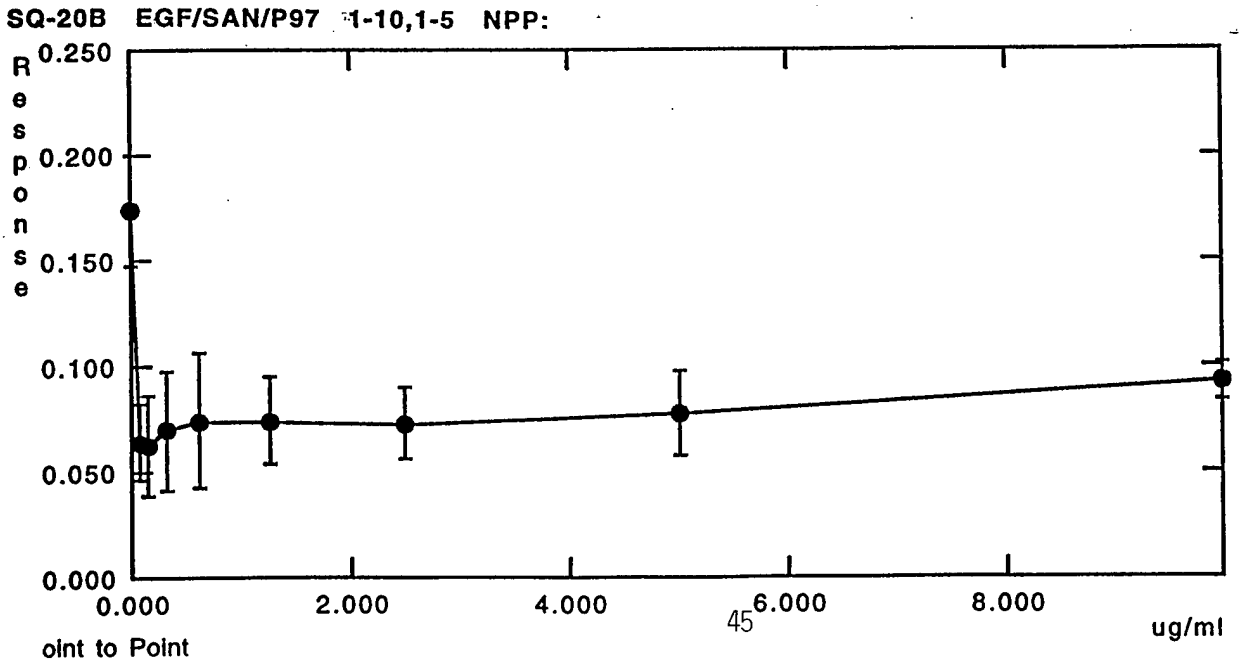
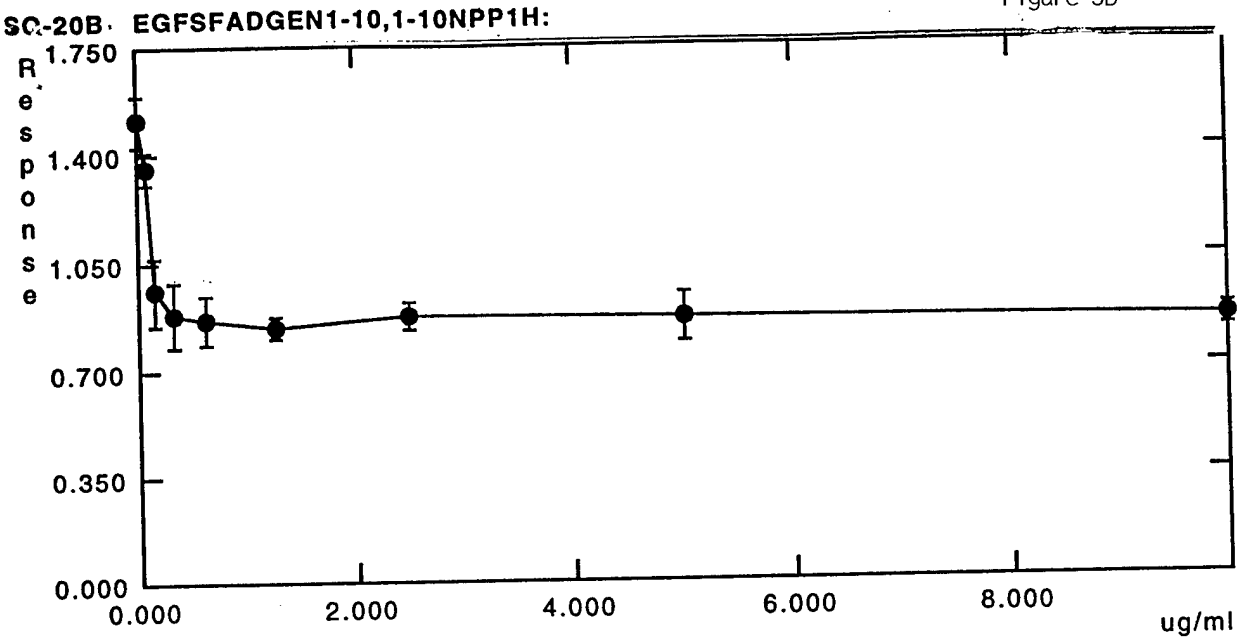
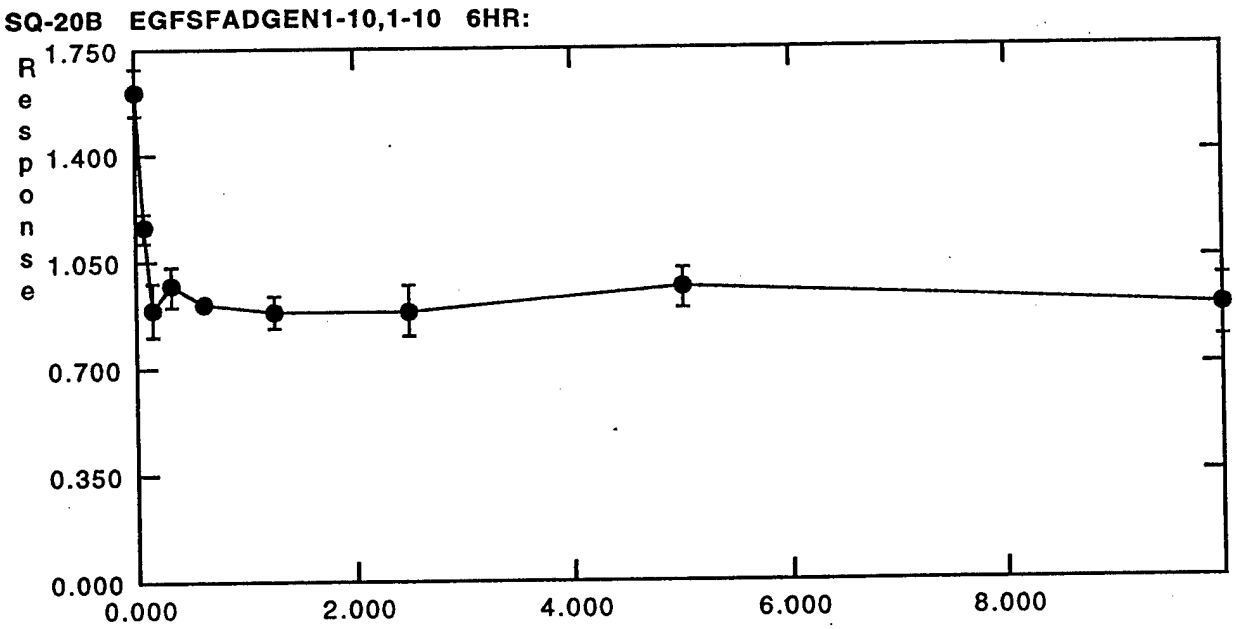
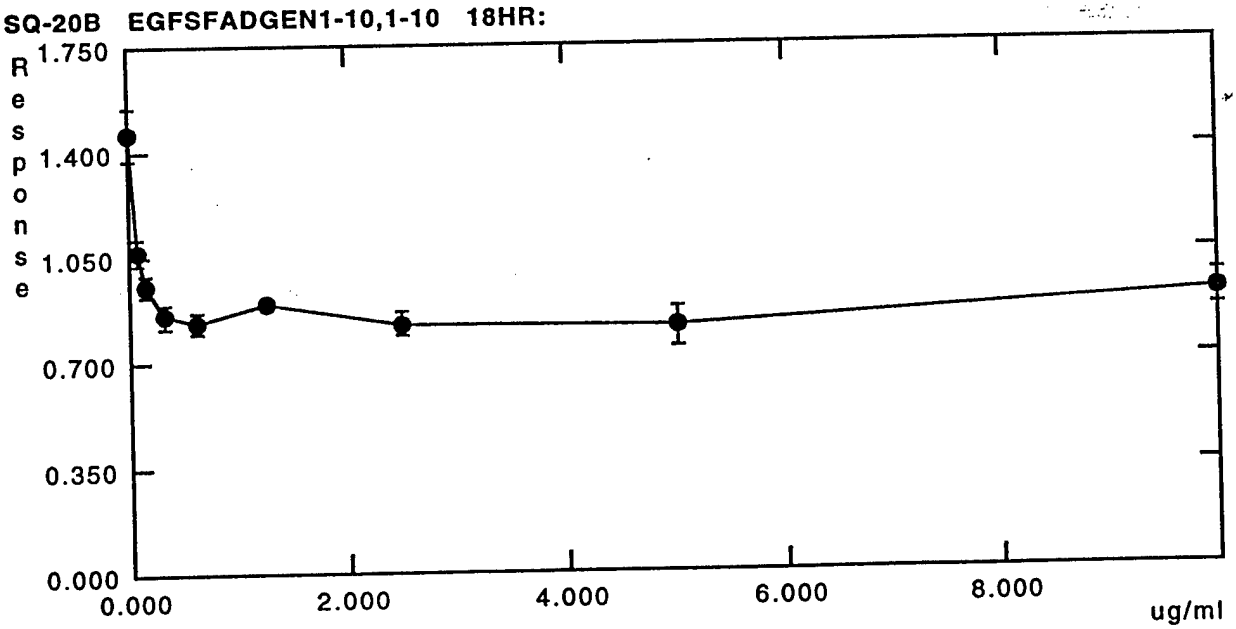

SQ-20B EGF/SAN/P97 1-10,1-5 NPP:
Response
0.250
0.200
0.150
0.100
0.050
0.000
0.000
2.000
4.000
6.000
8.000
ug/ml
oint to Point

Figure 5B

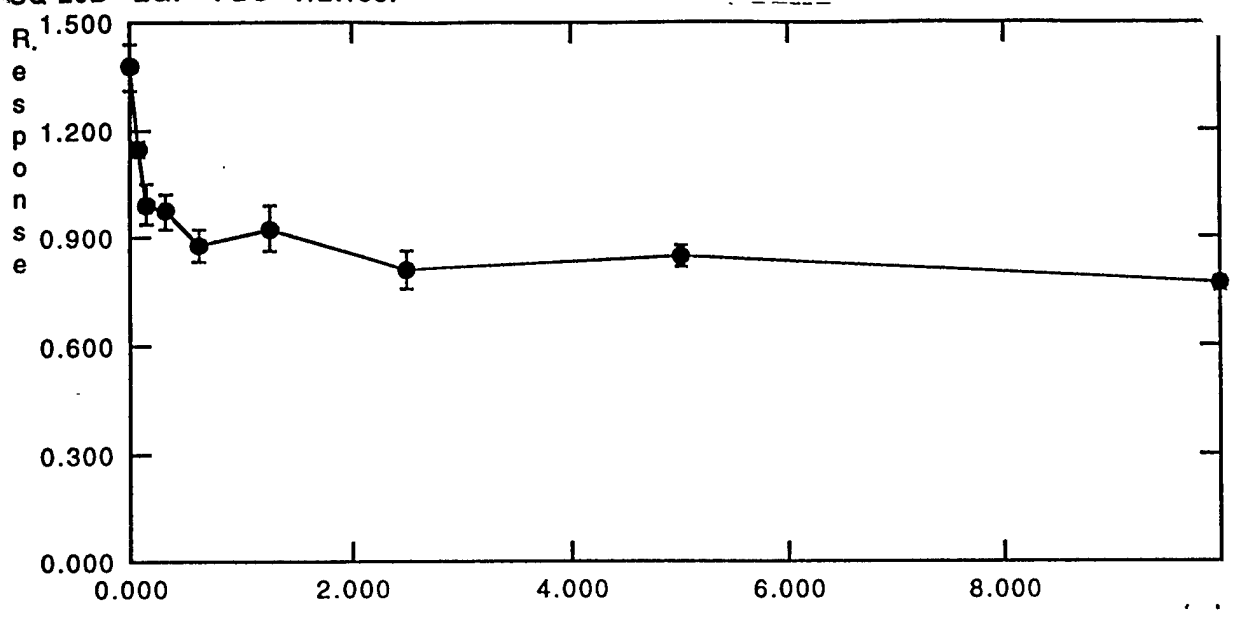


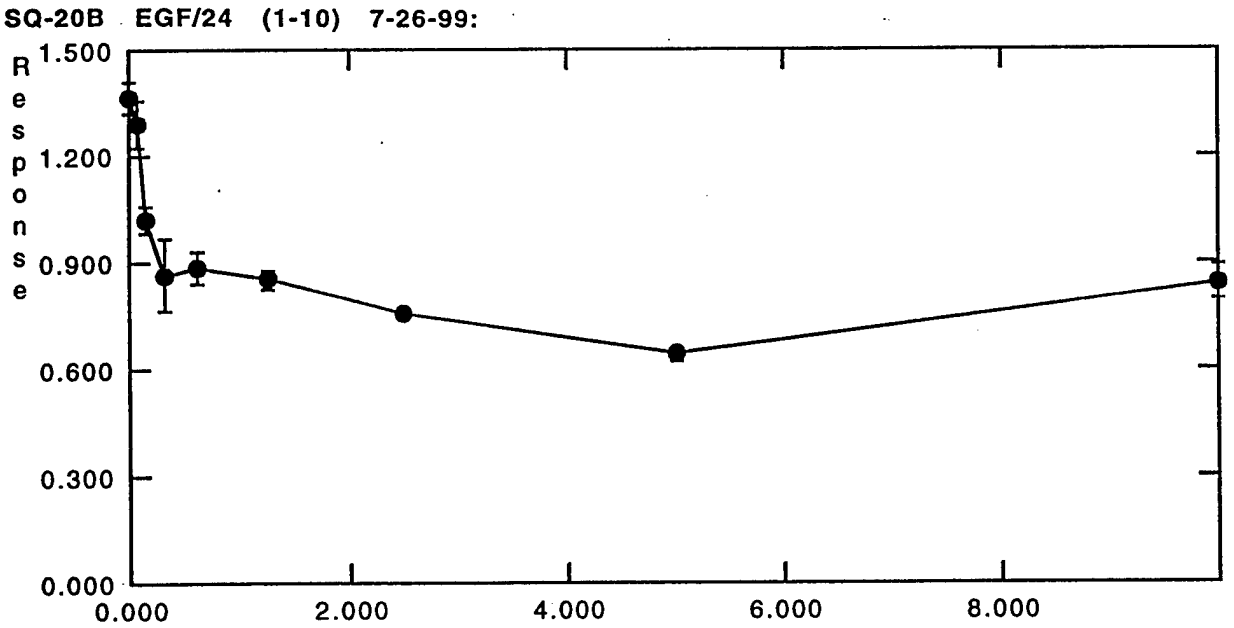


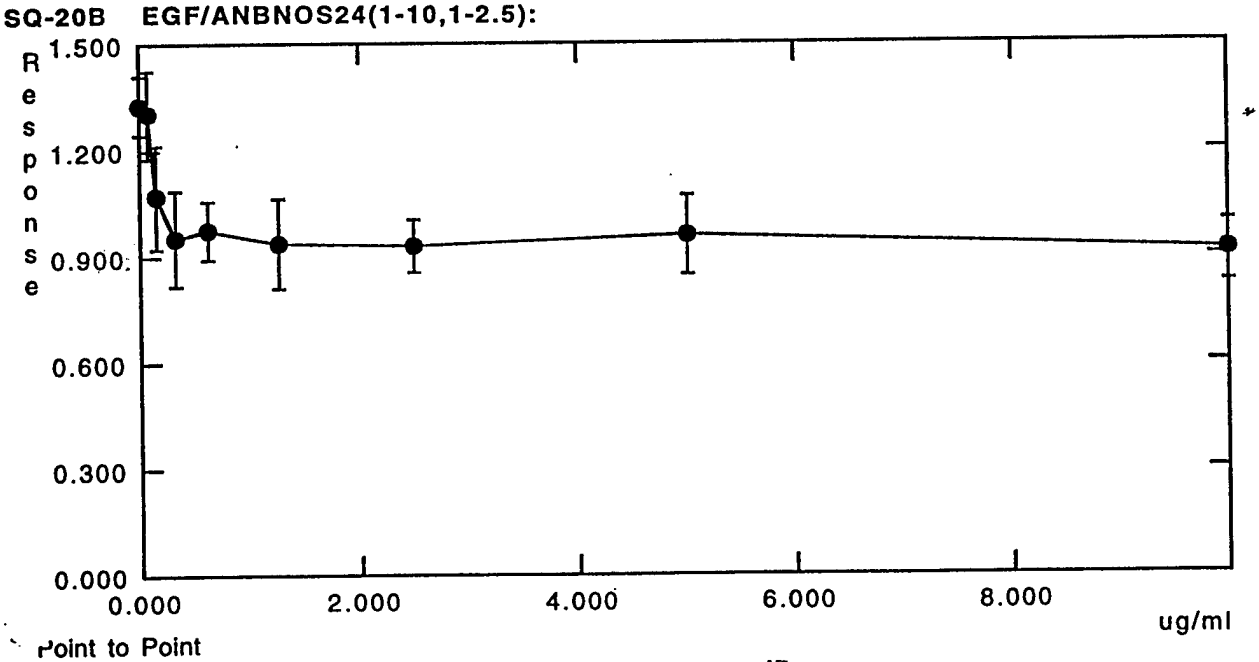


oint to Point

Figure 5C

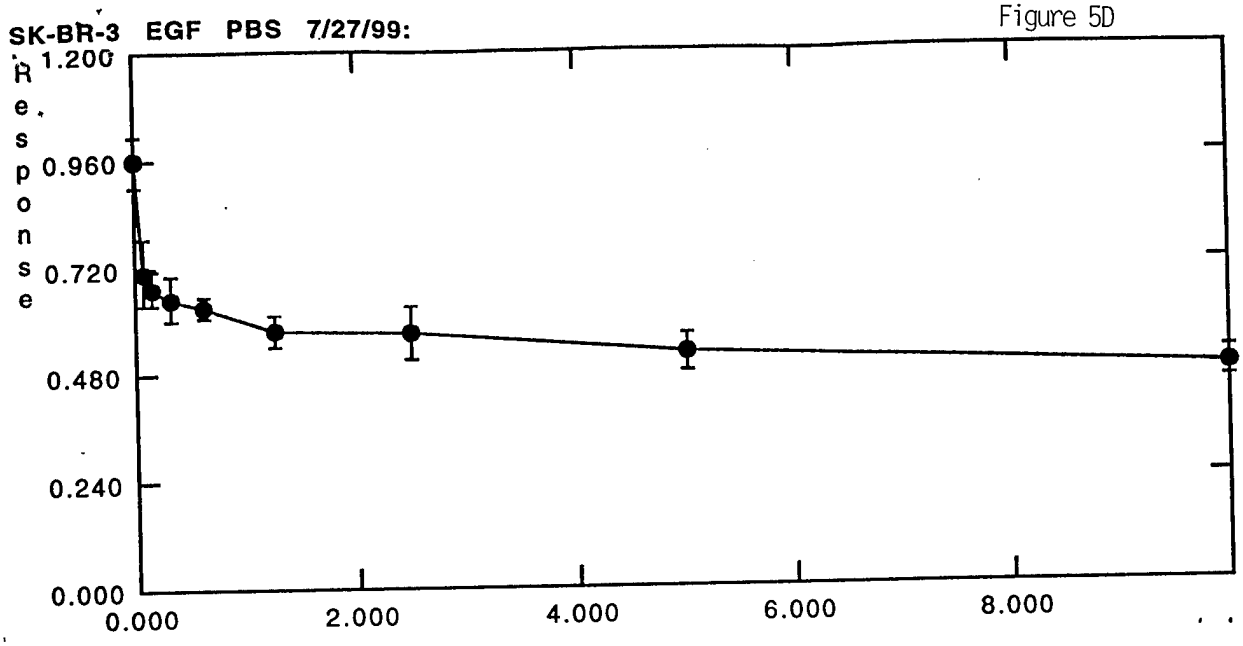


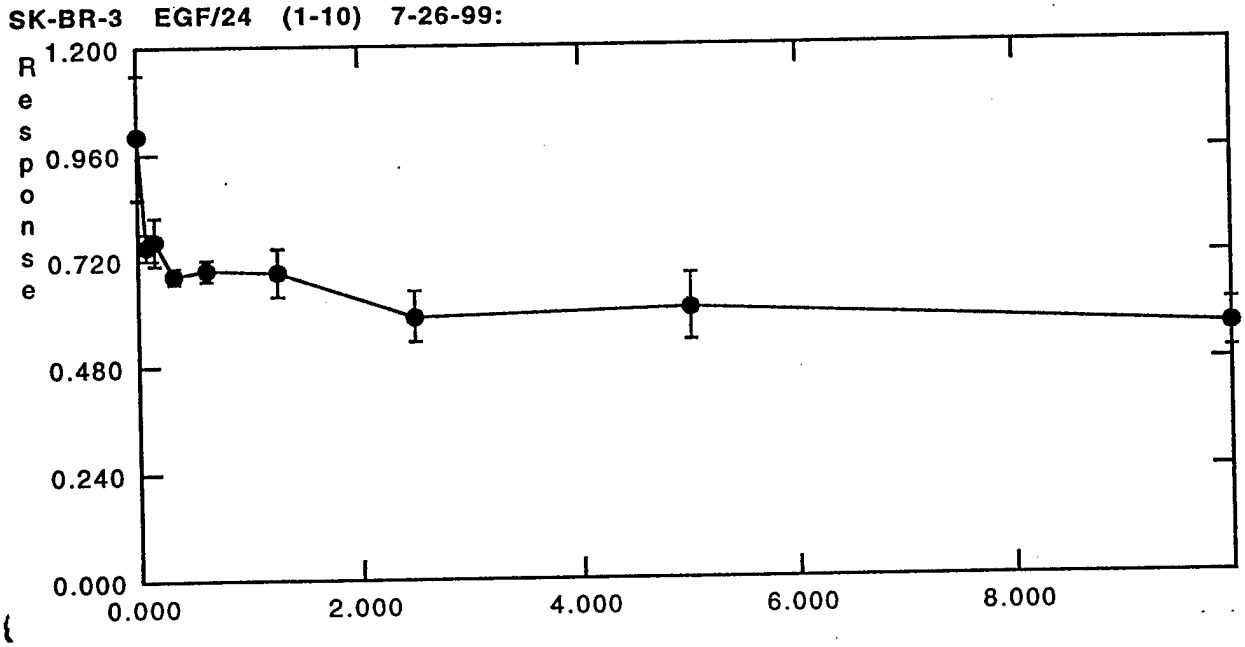


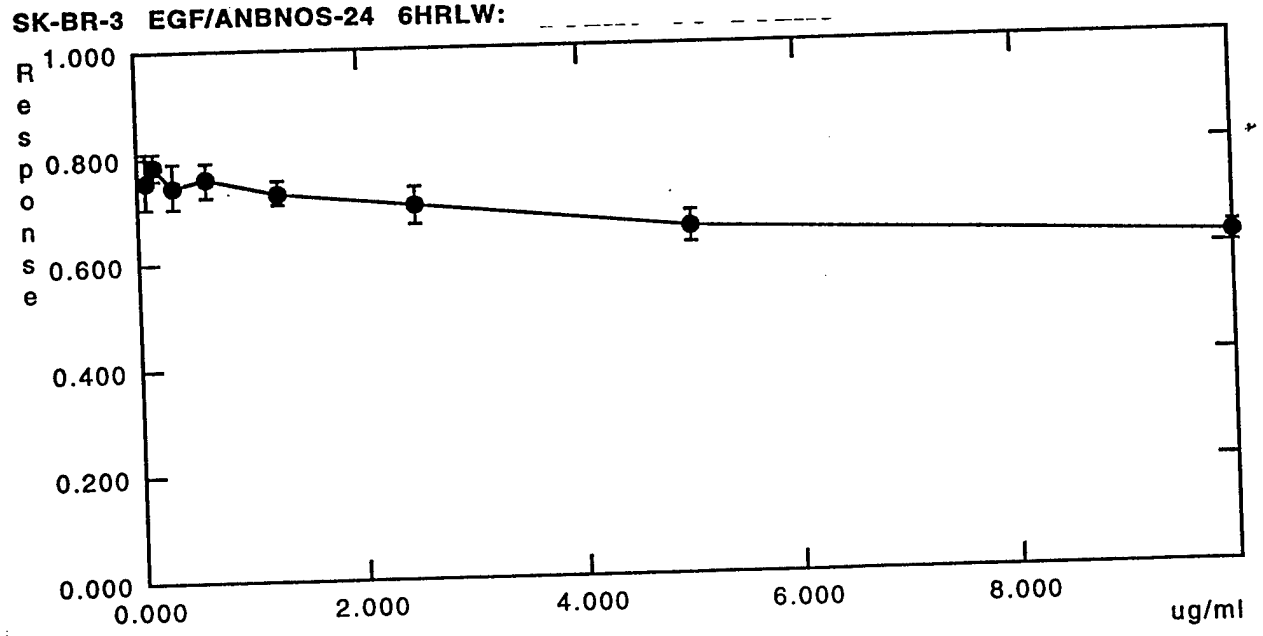


Point to Point

Figure 5D







Point to Point

Appendix II

Monkey 68-K

Animal Identification and Necropsy Number: 68-K

GROSS OBSERVATIONS:

Integumentary:	No gross lesions (NGL)
Cardiovascular:	There are hemorrhages in the subcutis adjacent to venipuncture sites.
Respiratory:	NGL
Alimentary:	NGL
Urinary:	NGL
Genital:	NGL
Hemolymphopoietic:	NGL
Endocrine:	NGL
Musculoskeletal:	There are injection sites in the skeletal muscle of the thighs.
Nervous and special senses	NGL

HISTOPATH OBSERVATIONS: Pending

Animal Identification: 68-K
Species: M. fasciculata

Tissues submitted for Histopathology:

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
31.

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

HISTOPATHOLOGY REPORT

DATE OF REPORT	9/17/99	**AGE**	adult
DATE OF NECROPSY	9/30/98	**SEX**	f
NECROPSY NUMBER	68-K	**SUPPLIER**	
INVESTIGATOR	Gunther	**PATHOLOGIST**	R. Gunther
DEPARTMENT	RAR	**PM INTERVAL**	>30 min
SPECIES	M. fasciculata	**LAB NUMBER**	
BREED/STRAIN		**LAB TESTS**	
ANIMAL ID	68-K	**NO. of ANIMALS**	1

HISTORY: Test-article toxicity study

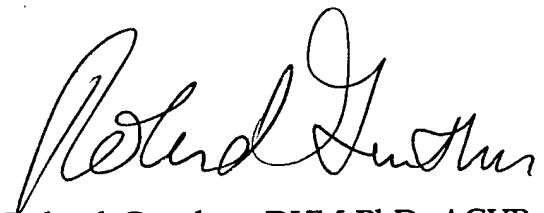
KEYWORDS: Genistein, epidermal growth factor

FINAL DIAGNOSES: No evidence of test article-related deleterious effects

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant lesions.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: **68-K**

HISTOPATH OBSERVATIONS::

Integumentary:	There are no significant lesions (NSL) in multiple sections of skin, lip and eyelid.
Cardiovascular:	NSL- heart, aorta
Respiratory:	NSL- lung, trachea
Alimentary:	NSL- tongue, esophagus, stomach, large and small intestine, liver, pancreas
Urinary:	NSL- kidneys, urinary bladder
Genital:	NSL- ovary, uterus
Hemolymphopoietic:	NSL- spleen, bonemarrow, lymph nodes
Endocrine:	NSL-pancreas, thyroid, adrenal
Musculoskeletal:	NSL- skeletal muscle, bone
Nervous and special senses	NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

Monkey 68-I

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

GROSS REPORT

DATE OF REPORT	10/1/98	**AGE**	adult
DATE OF NECROPSY	9/30/98	**SEX**	f
NECROPSY NUMBER	68-I	**SUPPLIER**	
INVESTIGATOR	Gunther	**PATHOLOGIST**	R. Gunther
DEPARTMENT	RAR	**PM INTERVAL**	>30 min
SPECIES	M. fasciculata	**LAB NUMBER**	
BREED/STRAIN		**LAB TESTS**	
ANIMAL ID	68-I	**NO. of ANIMALS**	1

HISTORY: test-article toxicity study

KEYWORDS: genistein, epidermal growth factor

FINAL DIAGNOSES: pending histopathologic evaluation

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant gross lesions.

Pathologist:

Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: 68-I

GROSS OBSERVATIONS:

Integumentary:	No gross lesions (NGL)
Cardiovascular:	There are hemorrhages in the subcutis adjacent to venipuncture sites.
Respiratory:	NGL
Alimentary:	NGL
Urinary:	NGL
Genital:	NGL
Hemolymphopoietic:	NGL
Endocrine:	NGL
Musculoskeletal:	There are injection sites in the skeletal muscle of the thighs.
Nervous and special senses	NGL

Animal Identification: 68-I
Species: M. fasciculata

Tissues submitted for Histopathology:

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
31.

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

HISTOPATHOLOGY REPORT

DATE OF REPORT	9/17/99	**AGE**	adult
DATE OF NECROPSY	9/30/98	**SEX**	f
NECROPSY NUMBER	68-I	**SUPPLIER**	
INVESTIGATOR	Gunther	**PATHOLOGIST**	R. Gunther
DEPARTMENT	RAR	**PM INTERVAL**	>30 min
SPECIES	M. fasciculata	**LAB NUMBER**	
BREED/STRAIN		**LAB TESTS**	
ANIMAL ID	68-I	**NO. of ANIMALS**	1

HISTORY: test-article toxicity study

KEYWORDS: genistein, epidermal growth factor

FINAL DIAGNOSES: No evidence of test article-related deleterious effects

CAUSE OF DEATH: Euthanasia

COMMENTS: No significant lesions are found in any tissue.

Pathologist:

Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: 68-I

HISTOPATH OBSERVATIONS:

Integumentary:	There are no significant lesions (NSL) in multiple sections of skin, eyelid and lip.
Cardiovascular:	NSL-heart, aorta
Respiratory:	NSL- lung and trachea
Alimentary:	NSL- tongue, esophagus, stomach, large and small intestine, liver, pancreas
Urinary:	NSL- kidneys, urinary bladder
Genital:	NSL- ovary, uterus
Hemolymphopoietic:	NSL- spleen, lymph nodes, bonemarrow
Endocrine:	NSL- pancreas, adrenal, thyroid, parathyroid
Musculoskeletal:	NSL- skeletal muscle, rib
Nervous and special senses	NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

Monkey 68-N

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

GROSS REPORT

DATE OF REPORT	10/13/98	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-N	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-N	NO. of ANIMALS	1

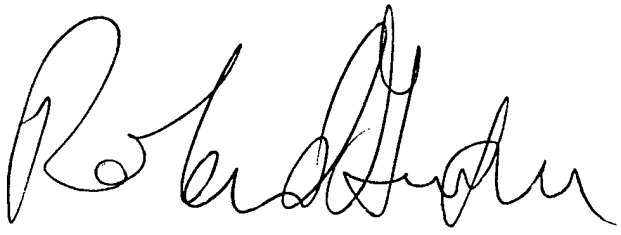
HISTORY: test-article toxicity study

KEYWORDS: genistein, epidermal growth factor

FINAL DIAGNOSES: pending histopathologic evaluation

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant gross lesions.

Pathologist: 

Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: 68-N

GROSS OBSERVATIONS:

Integumentary:	No gross lesions (NGL)
Cardiovascular:	There are hemorrhages in the subcutis adjacent to venipuncture sites.
Respiratory:	NGL
Alimentary:	NGL
Urinary:	NGL
Genital:	NGL
Hemolymphopoietic:	NGL
Endocrine:	NGL
Musculoskeletal:	There are injection sites in the skeletal muscle of the thighs.
Nervous and special senses	NGL

HISTOPATH OBSERVATIONS: Pending

Animal Identification: 68-N
Species: M. fasciculata

Tissues submitted for Histopathology:

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
31.

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

HISTOPATHOLOGY REPORT

DATE OF REPORT	9/17/99	**AGE**	adult
DATE OF NECROPSY	10/13/98	**SEX**	f
NECROPSY NUMBER	68-N	**SUPPLIER**	
INVESTIGATOR	Gunther	**PATHOLOGIST**	R. Gunther
DEPARTMENT	RAR	**PM INTERVAL**	>30 min
SPECIES	M. fasciculata	**LAB NUMBER**	
BREED/STRAIN		**LAB TESTS**	
ANIMAL ID	68-N	**NO. of ANIMALS**	1

HISTORY: Test-article toxicity study

KEYWORDS: Genistein, epidermal growth factor

FINAL DIAGNOSES: No evidence of test article-related deleterious effects

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant lesions.

Pathologist: 

Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: **68-N**

HISTOPATH OBSERVATIONS:

Integumentary:	There are no significant lesions (NSL) in multiple sections of skin, lip and eyelid.
Cardiovascular:	NSL- heart, aorta
Respiratory:	NSL-lung, trachea
Alimentary:	NSL- tongue, esophagus, stomach, large and small intestine, pancreas, liver
Urinary:	NSL- There is a mild increase in glomerular mesangial matrix. No lesions in the urinary bladder.
Genital:	NSL- ovary, uterus
Hemolymphopoietic:	NSL-lymph nodes, spleen, bonemarrow
Endocrine:	NSL-thyroid, parathyroid, pancreas, adrenal
Musculoskeletal:	NSL- skeletal muscle, bone
Nervous and special senses	NSL- multiple areas of brain and spinal cord, peripheral nerve, eye

Monkey 68-J

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

GROSS REPORT

DATE OF REPORT	10/13/98	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-J	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-J	NO. of ANIMALS	1

HISTORY: test-article toxicity study

KEYWORDS: genistein, epidermal growth factor

FINAL DIAGNOSES: pending histopathologic evaluation

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant gross lesions.

Pathologist: 

Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: 68-J

GROSS OBSERVATIONS:

Integumentary:	No gross lesions (NGL)
Cardiovascular:	There are hemorrhages in the subcutis adjacent to venipuncture sites.
Respiratory:	NGL
Alimentary:	NGL
Urinary:	NGL
Genital:	NGL
Hemolymphopoietic:	NGL
Endocrine:	NGL
Musculoskeletal:	There are injection sites in the skeletal muscle of the thighs.
Nervous and special senses	NGL

HISTOPATH OBSERVATIONS: Pending

Animal Identification: 68-J
Species: M. fasciculata

Tissues submitted for Histopathology:

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
31.

Animal Identification and Necropsy Number: 68-J

HISTOLOGIC OBSERVATIONS:

Integumentary:	There are no significant (NSL) lesions in multiple sections of skin, eyelid and lip.
Cardiovascular:	NSL- heart and aorta
Respiratory:	NSL- lung, trachea
Alimentary:	NSL- esophagus, tongue, stomach, large and small intestine, pancreas, liver
Urinary:	NSL- kidneys, urinary bladder
Genital:	NSL- ovary, uterus
Hemolymphopoietic:	NSL- spleen, lymph nodes, bonemarrow
Endocrine:	NSL-pancreas, adrenal, thyroid
Musculoskeletal:	NSL- skeletal muscle, bone
Nervous and special senses	NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

ANIMAL NECROPSY REPORT
RESEARCH ANIMAL RESOURCES
UNIT OF COMPARATIVE MEDICINE
ACADEMIC HEALTH SCIENCES
UNIVERSITY OF MINNESOTA

HISTOPATHOLOGIC REPORT

DATE OF REPORT	9/17/99	**AGE**	adult
DATE OF NECROPSY	10/13/98	**SEX**	f
NECROPSY NUMBER	68-J	**SUPPLIER**	
INVESTIGATOR	Gunther	**PATHOLOGIST**	R. Gunther
DEPARTMENT	RAR	**PM INTERVAL**	>30 min
SPECIES	M. fasciculata	**LAB NUMBER**	
BREED/STRAIN		**LAB TESTS**	
ANIMAL ID	68-J	**NO. of ANIMALS**	1

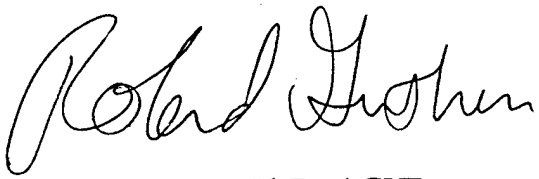
HISTORY: test-article toxicity study

KEYWORDS: genistein, epidermal growth factor

FINAL DIAGNOSES: No evidence of test article-related deleterious effects

CAUSE OF DEATH: Euthanasia

COMMENTS: There are no significant lesions.

Pathologist: 

Roland Gunther, DVM-PhD, ACVP

Appendix III

EXPER DATE	MS #	TREATMENT	DEATH DATE	GRP	SURV	D/S	HISTO	BEG WT (g)	END WT (g)	Chg Wt (g)	Mean Wt Chg (g)	SEM	Median Chg (g)
4/19/99	27451	Taxol	5/19/99	1	30	SH	X	18.5	21.7	3.2	3.55	0.35	3.55
4/19/99	27452	Taxol	5/19/99	1	30	SH	X	16.1	20	3.9			
4/19/99	27453	Taxol + EGF-Gen	5/19/99	2	30	SH	X	17	19.2	2.2	3.7	1.5	3.7
4/19/99	27454	Taxol + EGF-Gen	5/19/99	2	30	SH	X	13.6	18.8	5.2			
4/19/99	27455	CTX + EGF-Gen	5/19/99	3	30	SH	X	17.4	21.1	3.7	2.2	1.5	2.2
4/19/99	27456	CTX + EGF-Gen	5/19/99	3	30	SH	X	20.5	21.2	0.7			
4/19/99	27457	Adriamycin	4/27/99	4	8	D	X	19	13.5	-5.5	-5.8	0.3	-5.8
4/19/99	27458	Adriamycin	4/28/99	4	9	D	X	18.1	12	-6.1			
4/19/99	27459	Adriamycin + EGF-Gen	4/24/99	5	5	D	X	15.3	11.5	-3.8	-4.65	0.85	-4.65
4/19/99	27460	Adriamycin + EGF-Gen	4/26/99	5	7	D	X	19.1	13.6	-5.5			
4/19/99	27461	MTX	5/19/99	6	30	SH	X	17	21.1	4.1	3.25	0.85	3.25
4/19/99	27462	MTX	5/19/99	6	30	SH	X	19.7	22.1	2.4			
4/19/99	27463	MTX + EGF-Gen	5/19/99	7	30	SH	X	18.5	21.8	3.3	2.8	0.5	2.8
4/19/99	27464	MTX + EGF-Gen	5/19/99	7	30	SH	X	18.4	20.7	2.3			

Vendor: Taconic Strain: SCID Age: 5 weeks Sex: F Average Weight: 17.7g

Drug: Taxol Doses: 17 mg/kg/d (0.301 mg/ms/d) x 5d Method of Injection: IP

Drug: Cytoxan (CTX) Doses: 50 mg/kg/d (0.885 mg/ms/d) x 2d Method of Injection: IP

Drug: Adriamycin Doses: 8 mg/kg/d (0.142 mg/ms/d) x 1d Method of Injection: IP

Drug: Methyltrexate (MTX) Doses: 1 mg/kg/d (0.018 mg/ms/d) x 5d Method of Injection: IP

Drug: EGF-Gen Doses: 4 µg/d x 10d (starts day following last chemotherapy treatment for the drug treating with)

Method of Injection: IP

# of Mice/Group: 2 Volume Injected: 0.2 ml Length of Study: 30d (May 19, 1999)

DIRECTIONS: Weigh mice prior to treatment.

Following sacrifice, weigh mice. Dissect all mice at time of sacrifice.

Dispose of carcass. Histologically process all tissues.

IN VIVO TOXICITY OF CHEMOTHERAPY DRUGS +/- EGF-GEN (4/19/99)

MATERIALS AND METHODS

Toxicity Studies in SCID Mice. All SCID mice used in this toxicity study were obtained from the specific pathogen free (SPF) breeding facilities of Taconic at 4 weeks of age. The mice were housed in the animal housing facility of the Hughes Institute. All husbandry and experimental contact made with the mice maintained SPF conditions. The mice were kept in microisolator cages (Lab Products, Inc., Maywood, NY) containing autoclaved food, water and bedding.

In this toxicity study, 14 weighed five week old female SCID mice averaging 17.7 g were administered intraperitoneal bolus injections of one of four chemotherapy drugs in 0.2 mL sterile water solution. Groups of 4 mice received treatments of one of the following: 17 mg/kg/d x 5d Taxol (0.34 mg/ms/d x 5d), 8 mg/kg/d x 1d Adriamycin (0.16 mg/ms/d), or 1 mg/kg/d x 5d Methyltrexate (0.02 mg/ms/d x 5d). Two mice from each of the aforementioned groups received 4 µg/d x 10d EGF-Gen starting the day following the last chemotherapy treatment for each drug. Two additional mice received 50 mg/kg/d x 2d Cytoxan (1 mg/ms/d x 2d). No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of day 30 LD_{50} values. At time of sacrifice or death, mice will be weighed. Multiple organs were collected within 4 hours after death, grossly examined, and processed for histopathologic examination. Mice surviving 30 days post-treatment were sacrificed and the tissues were immediately collected and preserved in 10% neutral phosphate buffered formalin.

RESULTS

There were no immediate adverse affects observed following drug administration. All surviving mice will be electively sacrificed healthy on day 30 (May 19, 1999).

All mice receiving Adriamycin, either alone or in combination with EGF-Gen, died between days 5 - 9. Refer to the experiment table for gross observations taken during dissection. One CTX + EGF-Gen mouse is showing some signs of toxicity (#27456) - scruffy coat, slowed movement. It will be closely examined throughout the weekend.

(5/14/99) Mice appear healthy. Mouse #27456 appears to have recovered from the earlier signs of toxicity. All remaining survivors will be sacrificed on Wednesday, May 19, 1999 (day 30).

(5/19/99) All surviving mice were sacrificed healthy on day 30, May 19, 1999. All mice were found to be unremarkable at time of sacrifice, with the exception of #27452 (Taxol) and #27464 (MTX + EGF-Gen). Gross observations are noted in the attached experiment table.

The mean experimental weight change observed in the Adriamycin and Adriamycin + EGF-Gen groups were -32.8% and -26.3%, respectively. For the five treatment groups that did not sustain any deaths during the experiment, the weight change ranged from 12.4% in the CTX + EGF-Gen group to 20.9% in the Taxol + EGF-Gen group.

Table 1. Life-Table Analysis of Survival Data and Statistical Analysis of Weight Change Following Chemotherapy +/- EGF-Gen Intraperitoneal Administration - 4/19/99

Treatment Group	# of Mice	Proportion Surviving (%) Day 15	Day 30	Survival p-value Taxol	Taxol + EGF-Gen	CTX + EGF-Gen	Adriamycin	Adriamycin + EGF-Gen	MTX	MTX + EGF-Gen
Taxol	2	100 ± 0.0	100 ± 0.0		NA	NA	0.1797	0.1797	NA	NA
Taxol + EGF-Gen	2	100 ± 0.0	100 ± 0.0	NA		NA	0.1797	0.1797	NA	NA
CTX + EGF-Gen	2	100 ± 0.0	100 ± 0.0	NA	NA		0.1797	0.1797	NA	NA
Adriamycin	2	0 ± 0.0	0 ± 0.0	0.1797	0.1797	0.1797		0.1797	0.1797	0.1797
Adriamycin + EGF-Gen	2	0 ± 0.0	0 ± 0.0	0.1797	0.1797	0.1797	0.1797		0.1797	0.1797
MTX	2	100 ± 0.0	100 ± 0.0	NA	NA	NA	0.1797	0.1797		NA
MTX + EGF-Gen	2	100 ± 0.0	100 ± 0.0	NA	NA	NA	0.1797	0.1797	NA	

Treatment Group	# of Mice	Mean Weight Change (g) ± SEM	Weight Change p-value* Taxol	Taxol + EGF-Gen	CTX + EGF-Gen	Adriamycin	Adriamycin + EGF-Gen	MTX	MTX + EGF-Gen
Taxol	2	3.55 ± 0.35		0.9313	0.4732	0.0024	0.0123	0.7751	0.3441
Taxol + EGF-Gen	2	3.70 ± 1.50	0.9313		0.5528	0.0250	0.0401	0.8185	0.6266
CTX + EGF-Gen	2	2.20 ± 1.50	0.4732	0.5528		0.0347	0.0579	0.6045	0.7408
Adriamycin	2	-5.80 ± 0.30	0.0024	0.0250	0.0347		0.3302	0.0098	0.0046
Adriamycin + EGF-Gen	2	-4.65 ± 0.85	0.0123	0.0401	0.0579	0.3302		0.0224	0.0171
MTX	2	3.25 ± 0.85	0.7751	0.8185	0.6045	0.0098	0.0224		0.6929
MTX + EGF-Gen	2	2.80 ± 0.50	0.3441	0.6266	0.7408	0.0046	0.0171	0.6929	

*Weight p-value determined by unpaired t-test analysis. A p-value <0.05 was considered significant.

Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study.

Experiment Date: 4/19/99.

Date: 8/31/99

Barbara J. Waurzyniak, DVM, MS.
Veterinary Pathologist
Hughes Institute - PreClinical Laboratory
2680 Patton Road
Roseville, MN 55113
Phone: 651-604-9064
Fax: 651-604-9065

Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

A. MATERIAL AND METHODS:

1. The study was performed as follows:

Beginning on 4/19/99, 5 weeks old female SCID mice received the following treatments.

Group 1: **Taxol (TXL):** 17 mg/kg/day x 5 days.
Group 2: **Taxol (TXL):** 17 mg/kg/day x 5 days **+ EGF-GEN:** 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.
Group 3: **Cytoxan (CTX):** 50 mg / kg / day x 2 days **+ EGF-GEN:** 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.
Group 4: **Adriamycin (ADM):** 8 mg / kg x 1 day.
Group 5: **Adriamycin (ADM):** 8 mg / kg x 1 day **+ EGF-GEN:** 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.
Group 6: **Methyltrexate (MTX):** 1 mg / kg / day x 5 days.
Group 7: **Methyltrexate (MTX):** 1 mg / kg / day x 5 days **+ EGF-GEN:** 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.

The group size was 2 mice per group.
No untreated control mice were included in the experiment.

2. **TABLE 1: Mouse Identification and Treatment Table..... 4/19/99**

Group:	1	2	3	4	5	6	7
Treatment	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
Mouse ID#'s:	27451 27452	27543 27454	27455 27456	27457 27458	27459 27460	27461 27462	27463 27464
Total # of mice / group	2	2	2	2	2	2	2
# of mice examined	2	2	2	2	2	2	2

3. **Table 2:.....Outcome (Survival - days):**

Group #	Treatment	Survival (days)
Group 1:	Taxol (TXL)	2/2 (100%) SH at 30 days.
Group 2:	Taxol (TXL)+ EGF-GEN	2/2 (100%) SH at 30 days.
Group 3:	Cytoxan (CTX) + EGF-GEN	2/2 (100%) SH at 30 days.
Group 4:	Adriamycin (ADM)	2/2 (100%) died on day 8 and 9.
Group 5:	Adriamycin (ADM) + EGF-GEN	2/2 (100%) died on day 5 and 7.
Group 6:	Methyltrexate (MTX)	2/2 (100%) SH at 30 days.
Group 7:	Methyltrexate (MTX) + EGF-GEN	2/2 (100%) SH at 30 days.

3. Clinical Phase, Necropsy and harvesting of tissues:

a. The clinical phase, necropsy and harvesting of tissues was performed at the Hughes Institute, 2680 Patton Road, Roseville, MN 55113.

b. At death, all mice had routine postmortem examinations. Tissues from selected mice were collected, fixed in 10% formalin, and processed for histologic sectioning in a routine manner. The histology slides were stained with Hematoxylin and Eosin.

c. The histologic evaluation of the tissues and report compilation was done by Barbara J. Waurzyniak, DVM., MS., (veterinary pathologist).

Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

B. EXPERIMENTAL RESULTS:

1. Potential test-agent related lesions (See Table 3):

   a. Bone Marrow, erythroid, myeloid and megakaryocytic depletion (pancytopenia), marked. Present in 2/2 (100%) of the mice in Group 4 (Adriamycin) and 2/2 (100%) of the mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.

   b. Stomach, focal gastric ulceration and inflammation. Present in 1/2 (50%) of the mice in Group 4 (Adriamycin).

   c. Kidney, acute tubular necrosis, mild, multifocal, renal cortex. Present in 2/2 (100%) of the mice in Group 4 (Adriamycin) and 2/2 (100%) of the mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.

   d. Liver, multifocal hepatic necrosis, mild, acute. Present 1/2 (50%) of the mice in Group 5 (Adriamycin + EGF-GEN). May be caused by Adriamycin or Mouse Hepatitis Virus.
   Hepatic necrosis was considered unlikely to be caused by EGF-GEN because of the absense of hepatic necrosis in Group 2 (Taxol + EGF-GEN), Group 3 (Cytoxan + EGF-GEN) and Group 7 (Methyltrexate + EGF-GEN).

   e. Ovary, follicular atrophy, moderate to marked. Present in 1/1 (100%) of the examined mice in Group 4 (Adriamycin) and 1/1 (100%) of the examined mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.

   f. Spleen, reduced hematopoiesis in the red pulp. Present in 1/1 (100%) of the examined mice in Group 4 (Adriamycin) and 2/2 (100%) of the examined mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.

2. Incidental findings:

   a. Heart, dystrophic epicardial mineralization and fibrosis, multifocal, mild to moderate. Present in :
   1/2 (50%) of mice in Group 3 (Cytoxan + EGF-Gen),
   1/2 (50%) of mice in Group 4 (Adriamycin),
   1/2 (50%) of mice in Group 5 (Adriamycin + EGF-Gen),
   1/2 (50%) of mice in Group 6 (Methyltrexate),
   1/2 (50%) of mice in Group 7 (Methyltrexate + EGF-Gen).

   b. Ovary, ovarian tumor, (probable granulosa cell tumor). Present in 1/1 (100%) of the examined mice in Group 1 (Taxol).

   c. Thymus, thymic cysts, multifocal. Most likely are developmental. Present in 1/1 (100%) of the examined mice in Group 1 (Taxol).

C. COMMENTS:

Based on the results of the histologic evaluation of the test animals, it is concluded that EGF-GEN is non-toxic under the conditions of this study.

Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

TABLE 2: Histopathologic Results from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

GROUP:	1	2	3	4	5	6	7
TREATMENT:	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
TISSUE / DIAGNOSIS / MODIFIER(S):							
BONE & BONE MARROW:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Erythroid, myeloid and megakaryocytic depletion, marked.	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
BRAIN:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
GUT:							
LARGE INTESTINE:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
SMALL INTESTINE:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
STOMACH:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Gastric ulcer, focally extensive, acute, with mild suppurative submucosal inflammation.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
HEART:							
1. WNL.	2/2 (100%)	2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Dystrophic mineralization and fibrosis, epicardium, multifocal, mild to moderate.	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
KIDNEY:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Tubular necrosis, acute, mild, multifocal, renal cortex.	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
LIVER:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Hepatic necrosis, mild, multifocal, acute.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)
LUNG:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)

Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

TABLE 2: Histopathologic Results from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.

GROUP:	1	2	3	4	5	6	7
TREATMENT:	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
TISSUE / DIAGNOSIS / MODIFIER(S):							
LYMPH NODE:							
1. WNL.	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)
2. NE.	1/2 (50%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)
OVARIES:							
1. WNL.	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	2/2 (100%)
2. NE.	1/2 (50%)	1/2 (50%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	0/2 (0%)
3. Follicular atrophy, moderate to marked.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	1/1 (100%)	0/2 (0%)	0/2 (0%)
4. Ovarian tumor, probable granulosa cell tumor.	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
PANCREAS:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
SKELETAL MUSCLE:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
SKIN:							
1. WNL.	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
SPINAL CORD:							
1. WNL.	1/1 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	1/2 (50%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
SPLEEN:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Reduced hematopoiesis, red pulp, moderate.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
THYMUS:							
1. WNL.	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	1/1 (100%)	2/2 (100%)	1/1 (100%)
2. NE.	1/2 (50%)	1/2 (50%)	0/2 (0%)	2/2 (100%)	1/2 (50%)	0/2 (0%)	1/2 (50%)
3. Cysts, multifocal, mild, (developmental).	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
URINARY BLADDER:							
1. WNL.	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
UTERUS:							
1. WNL.	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)
2. NE.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)

NOTES:

1. Normal mice may contain the following:
   a) hepatic sinusoidal inflammation, mild, multifocal consisting of a few small foci of macrophages, neutrophils, and/or lymphocytes;
   b) nonsuppurative cholangitis, mild;
   c) extramedullary hematopoiesis, mild multifocal.
   d) mild infiltrates of granulocytes in the gastric submucosa, particularly at the junction of the glandular and nonglandular regions.
   e) mild inflammation of the mesenteric fat and /or peritoneum.
1. WNL = Within Normal Limits.
2. NE = Not Examined.

Appendix IV

Proportion Surviving Tumor Free when treated with Chemotherapy Drugs in Combination with EGF-Gen administered against MDA MB 231 in SCID mice

Treatment Group	# of Mice	Proportion Surviving			Mean EFS (days)	Median EFS (days)	P-rank Value							
		15 Days	30 Days	45 Days			vs Control	vs EGF-Gen	vs Taxol	vs Adriamycin	vs Cytoxan	vs Taxol + EGF-Gen	vs Adriamycin + EGF-Gen	vs Cytoxan + EGF-Gen
Control	10	70 ± 14.5	60 ± 15.5	40 ± 15.5	26.2 ± 2.6	32		0.0218	0.6103	0.0745	0.4148	0.2622	0.1731	0.3743
EGF-Gen	10	30 ± 14.5	0 ± 0	0 ± 0	15.8 ± 1.7	15	0.0218		0.0051	0.2367	0.0663	0.0117	0.0382	0.0300
Taxol	10	100 ± 0	80 ± 12.6	50 ± 15.8	45.7 ± 4.0	44.5	0.6103	0.0051		0.0093	0.0801	0.0166	0.0244	0.0745
Adriamycin	10	70 ± 14.5	10 ± 9.5	0 ± 0	20.2 ± 2.8	18	0.0745	0.2367	0.0093		0.2604	0.0357	0.2135	0.0663
Cytoxan	10	70 ± 14.5	40 ± 15.5	20 ± 12.6	29.4 ± 5.2	23.5	0.4148	0.0663	0.0801	0.2604		0.9528	0.8590	0.9442
Taxol + EGF-Gen	10	90 ± 9.5	40 ± 15.5	10 ± 9.5	31.6 ± 4.3	27	0.2622	0.0117	0.0166	0.0357	0.9528		0.5940	0.9188
Adriamycin + EGF-Gen	10	60 ± 15.5	40 ± 15.5	10 ± 9.5	28.0 ± 5.2	21.5	0.1731	0.0382	0.0244	0.2135	0.8590	0.5940		0.6103
Cytoxan + EGF-Gen	10	80 ± 12.6	50 ± 15.8	30 ± 14.5	32.1 ± 5.2	28.5	0.3743	0.0300	0.0745	0.0663	0.9442	0.9188	0.6103	

This table is based on data collected up through day 71.

Doubling tumor progression survival of Chemotherapy compounds with EGF-Gen administered against MDA MB 231 in CB17 SCID mice

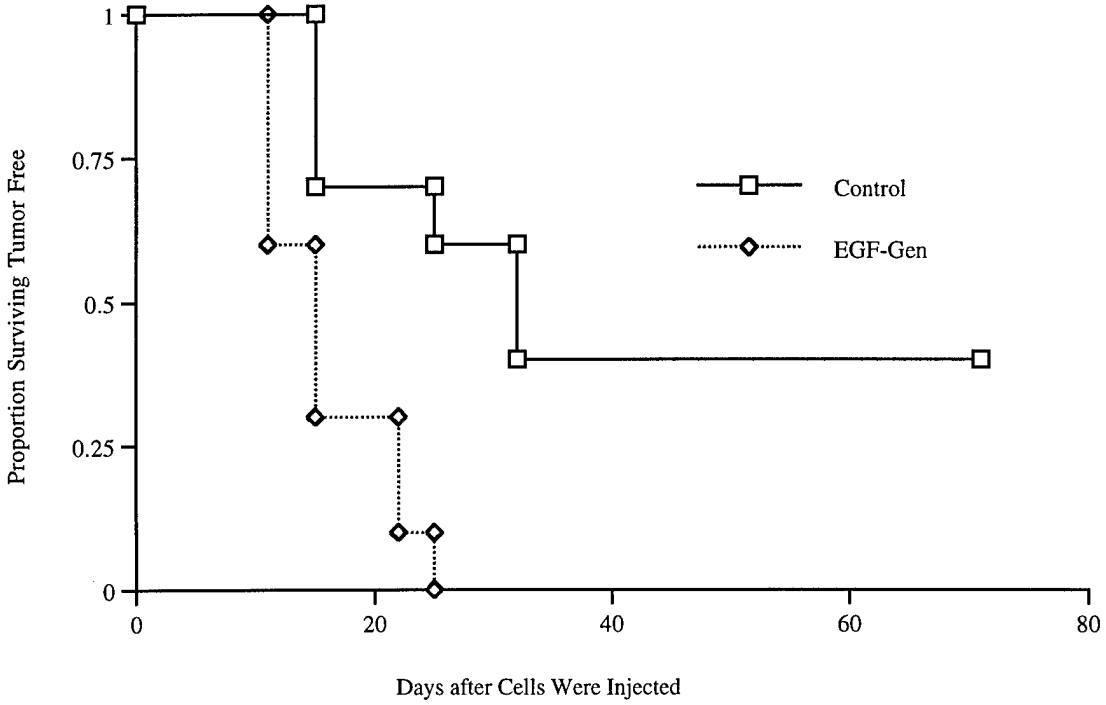
Treatment Group	# of Mice	Doubling Progression 30 Days	Doubling Progression 60 Days	Median PFS (days)	Mean PFS (days)	P-rank Value vs Control	vs EGF-Gen	vs Taxol	vs Adriamycin	vs Cytoxan	vs Taxol + EGF-Gen	vs Adriamycin + EGF-Gen	vs Cytoxan + EGF-Gen
Control	6	50 ± 20.4	0 ± 0	30.5	34.5 ± 5.0		0.7532	0.0277	0.0747	0.0431	0.1380	0.0464	0.0464
EGF-Gen	10	70 ± 14.5	10 ± 9.5	32	30.7 ± 2.4	0.7532		0.0166	0.0440	0.0209	0.0209	0.1097	0.0129
Taxol	10	100 ± 0	20 ± 12.6	53	56.8 ± 2.7	0.0277	0.0166		0.3454	0.7213	0.6744	0.0330	0.9594
Adriamycin	9	89 ± 10.5	11 ± 10.5	53	51.3 ± 4.3	0.0747	0.0440	0.3454		0.4412	0.7794	0.2936	0.1614
Cytoxan	10	100 ± 0	40 ± 15.5	56.5	54.4 ± 4.3	0.0431	0.0209	0.7213	0.4412		0.8590	0.0858	0.8785
Taxol + EGF-Gen	10	90 ± 9.5	30 ± 14.5	53	54.3 ± 4.5	0.1380	0.0209	0.6744	0.7794	0.8590		0.3454	0.7998
Adriamycin + EGF-Gen	9	89 ± 10.5	0 ± 0	46	45 ± 3.9	0.0464	0.1097	0.0330	0.2936	0.0858	0.3454		0.0117
Cytoxan + EGF-Gen	10	90 ± 9.5	30 ± 14.5	56.5	55.7 ± 5.1	0.0464	0.0129	0.9594	0.1614	0.8785	0.7998	0.0117	

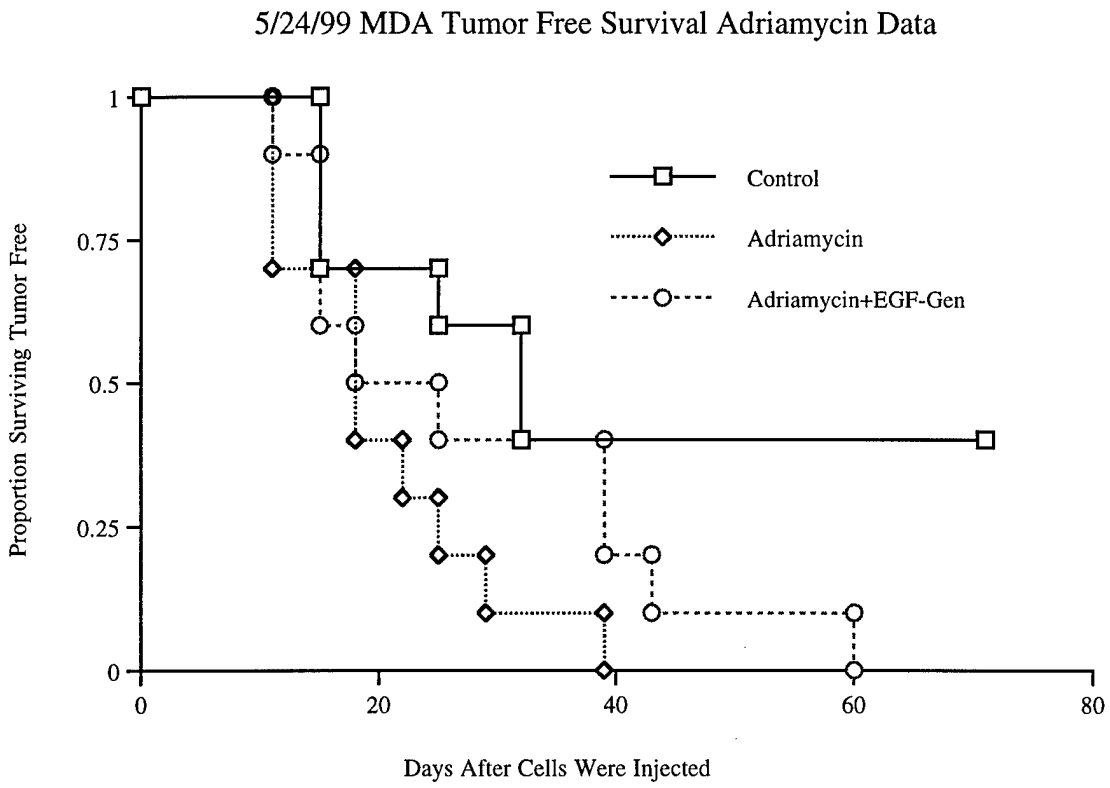
Tripling tumor progression survival of Chemotherapy compounds with EGF-Gen administered against MDA MB 231 in CB17 SCID mice

Treatment Group	# of Mice	Tripling Progression 30 Days	Tripling Progression 60 Days	Median PFS (days)	Mean PFS (days)	P-rank Value vs Control	vs EGF-Gen	vs Taxol	vs Adriamycin	vs Cytoxan	vs Taxol + EGF-Gen	vs Adriamycin + EGF-Gen	vs Cytoxan + EGF-Gen
Control	6	83 ± 15.2	0 ± 0	34	37.5 ± 5.7		0.8927	0.0431	0.0431	0.0464	0.1159	0.0464	0.0464
EGF-Gen	10	90 ± 9.5	10 ± 9.5	39	37.8 ± 2.0	0.8927		0.0144	0.0357	0.0069	0.0323	0.0910	0.0077
Taxol	10	100 ± 0	30 ± 14.5	60	58.9 ± 2.6	0.0431	0.0144		0.3105	0.2135	0.67444	0.0580	0.3270
Adriamycin	9	100 ± 0	22 ± 13.9	53	54.3 ± 3.1	0.0431	0.0357	0.3105		0.0440	0.8127	0.2626	0.0910
Cytoxan	10	100 ± 0	60 ± 15.5	67	63.3 ± 2.7	0.0464	0.0069	0.2135	0.0440		0.2340	0.0180	0.8385
Taxol + EGF-Gen	10	100 ± 0	30 ± 14.5	56.5	57.7 ± 3.2	0.1159	0.0323	0.6744	0.8127	0.2340		0.2049	0.1834
Adriamycin + EGF-Gen	9	100 ± 0	0 ± 0	50	50.0 ± 2.4	0.0464	0.0910	0.0580	0.2626	0.0180	0.2049		0.0117
Cytoxan + EGF-Gen	10	100 ± 0	50 ± 15.8	63.5	61.8 ± 2.1	0.0464	0.0077	0.3270	0.0910	0.8385	0.1834	0.0117	

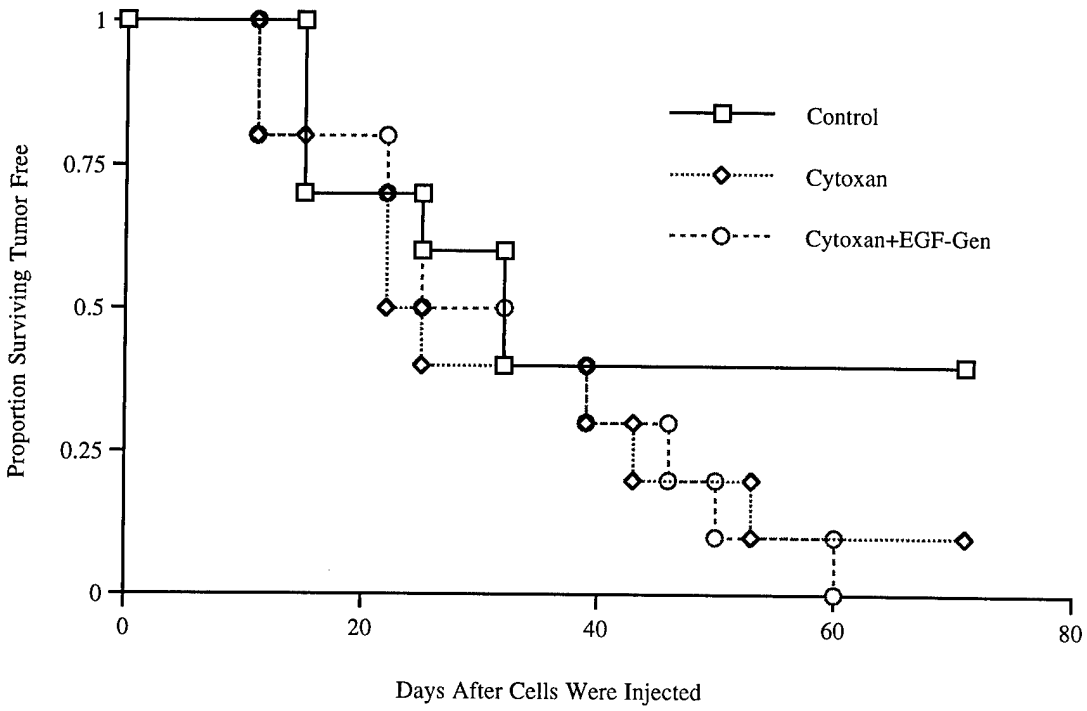
This table is based on data collected up through day 71.

5/24/99 MDA Tumor Free Survival EGF-Gen Data

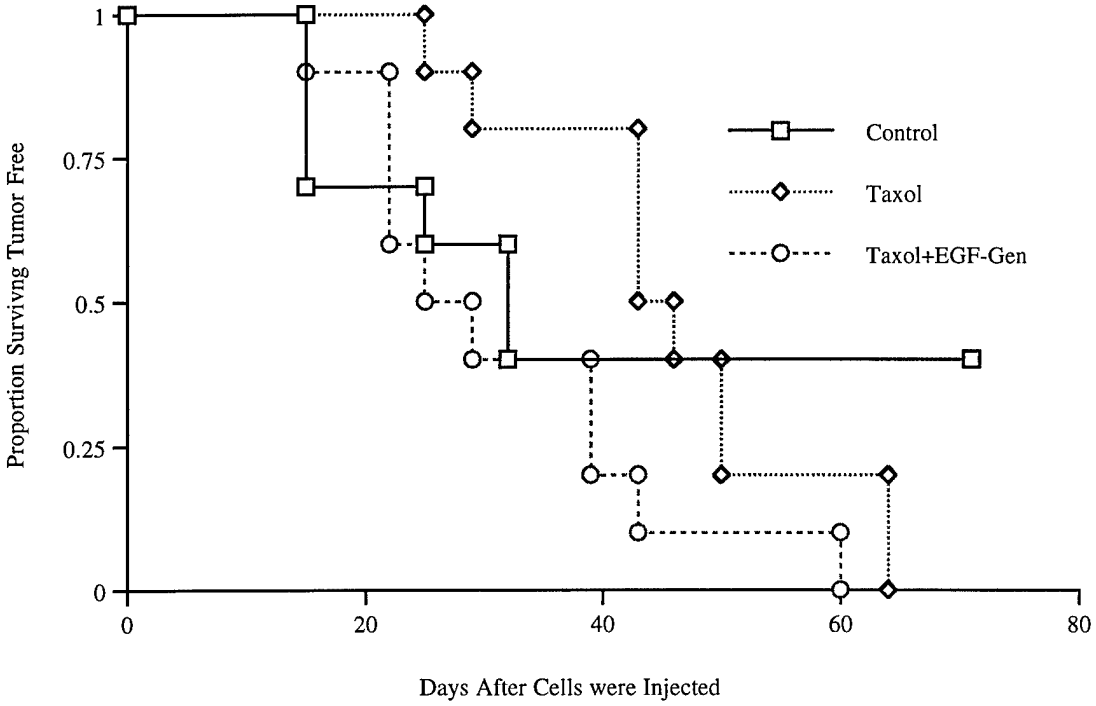



5/24/99 MDA Tumor Free Survival Adriamycin Data
Proportion Surviving Tumor Free
1
0.75
0.5
0.25
0
0
20
40
60
80
Days After Cells Were Injected
Control
Adriamycin
Adriamycin+EGF-Gen

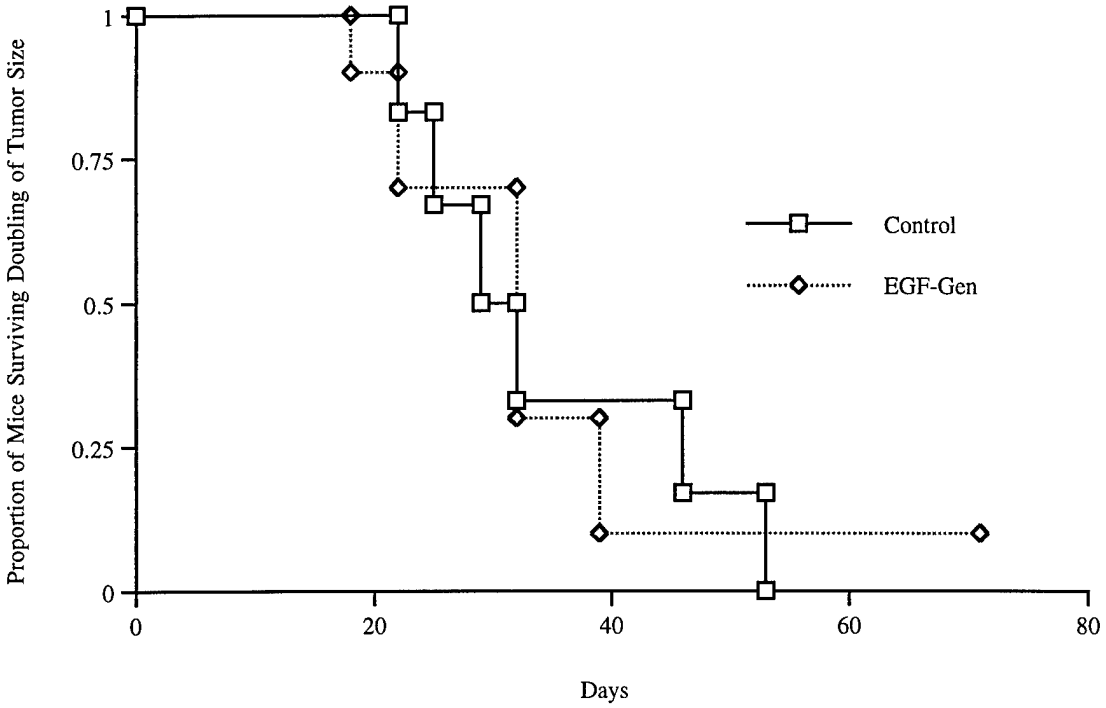
5/24/99 MDA Tumor Free Survival Cytoxan Data



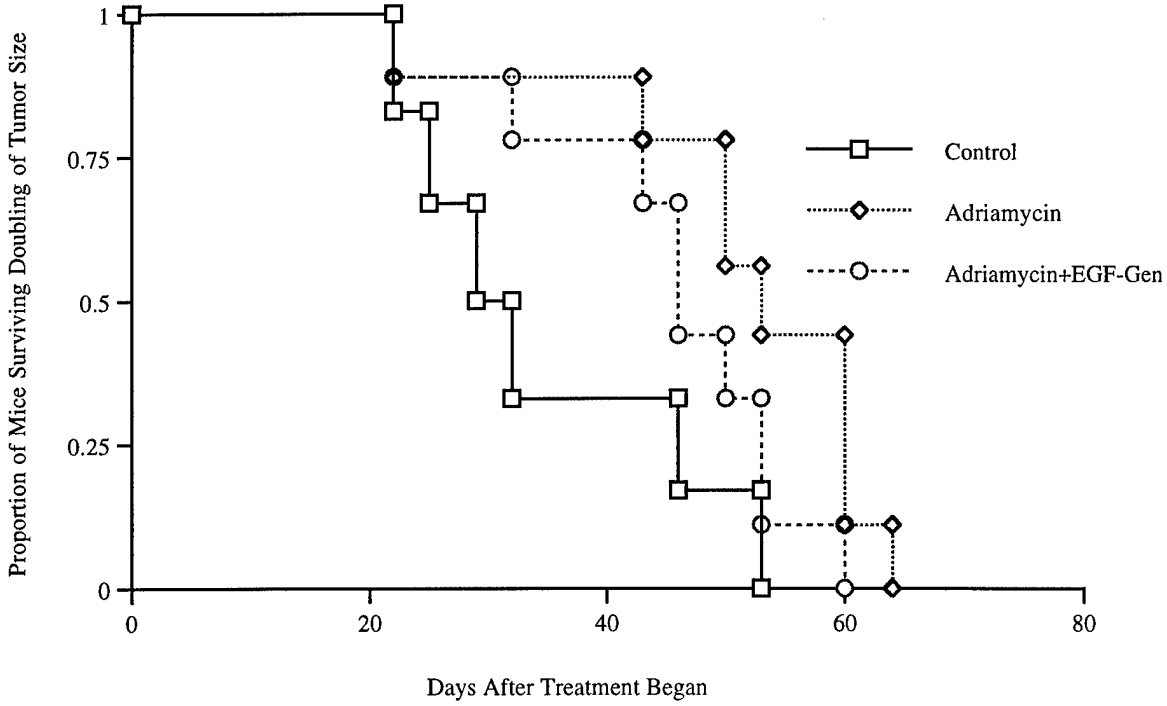
5/24/99 MDA Tumor Free Survival Taxol Data



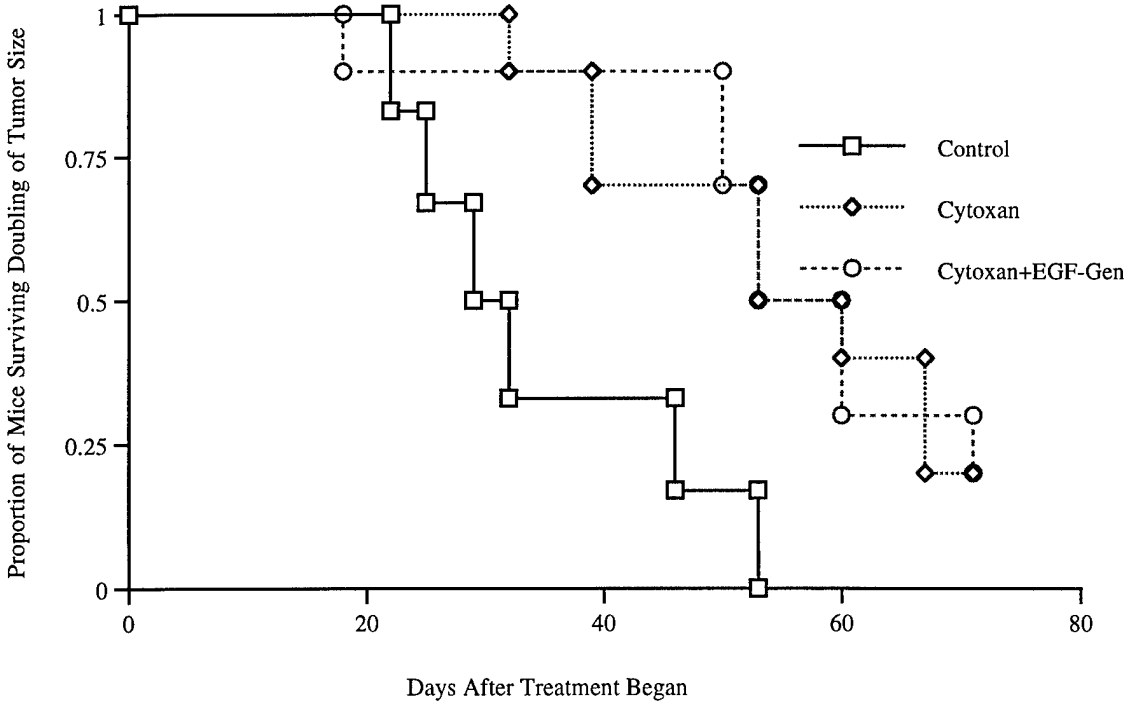
5/24/99 MDA Tumor Progression EGF-Gen Doubling Data



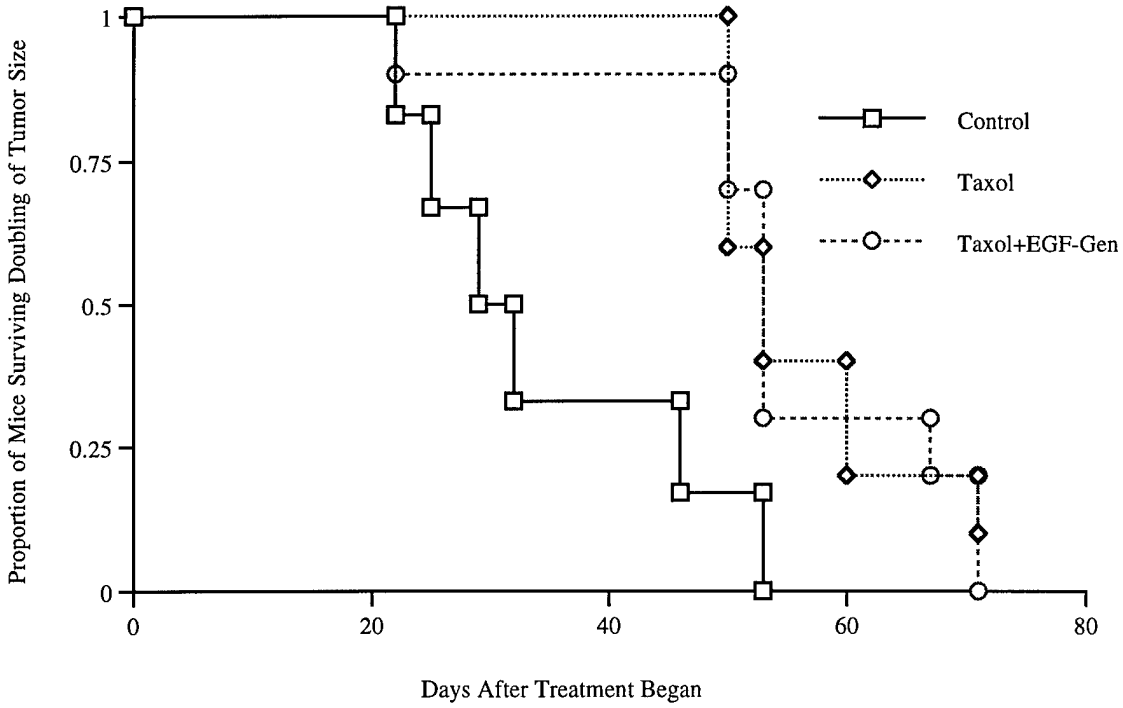
5/24/99 MDA Tumor Progression Adriamycin Doubling Data



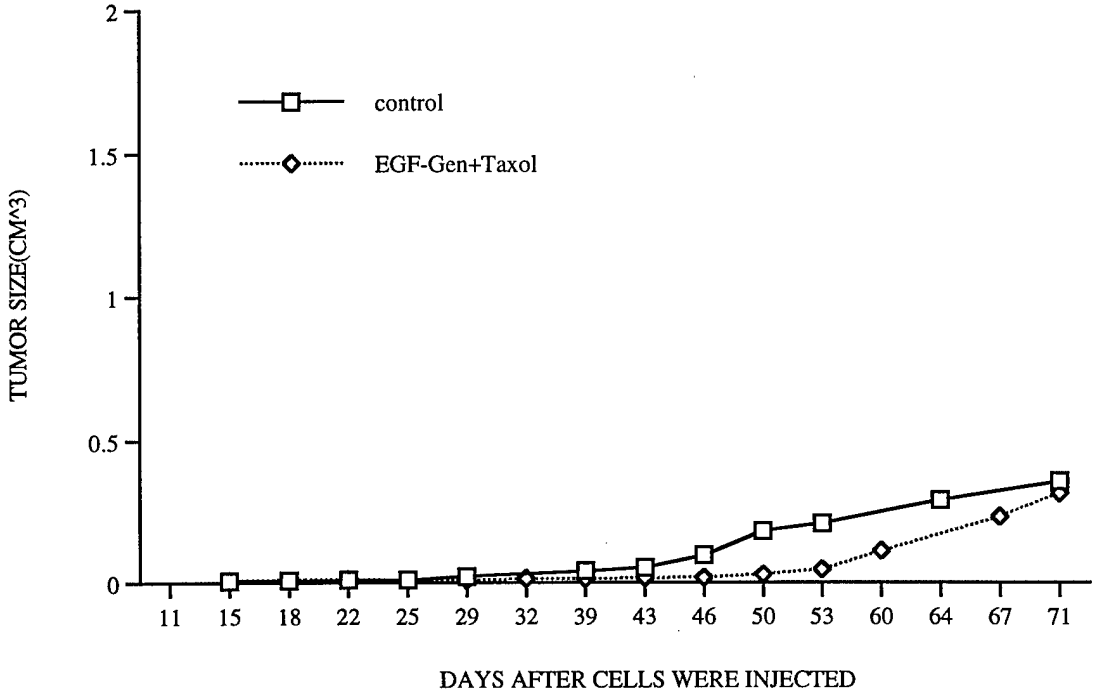
5/24/99 MDA Tumor Progression Cytoxan Doubling Data



5/24/99 MDA Tumor Progression Taxol Doubling Data

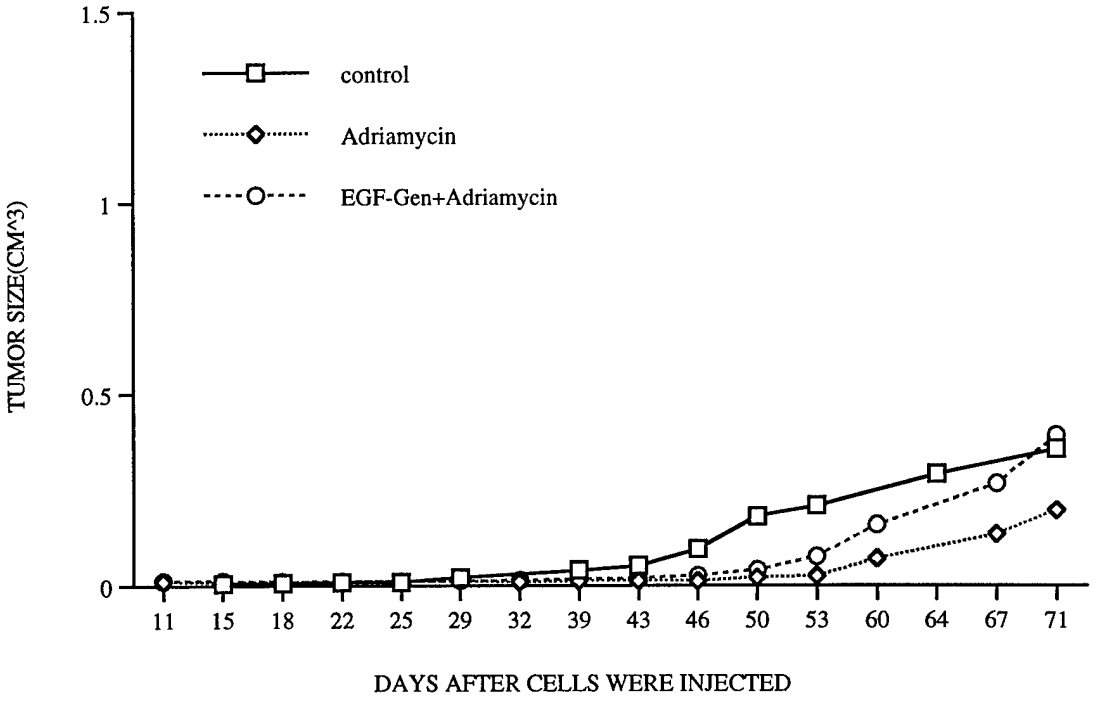


5/24/99 MDA EGF-Gen + Taxol DATA

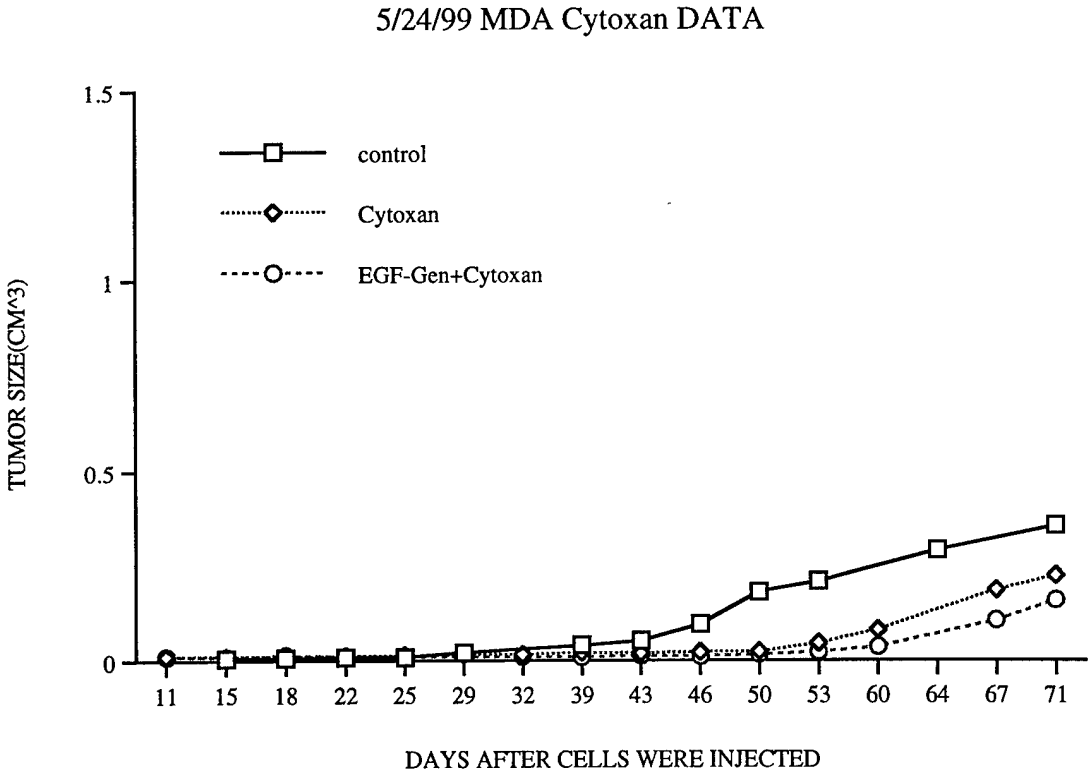


This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with a combination of EGF-Gen and Taxol.

5/24/99 MDA Adriamycin DATA

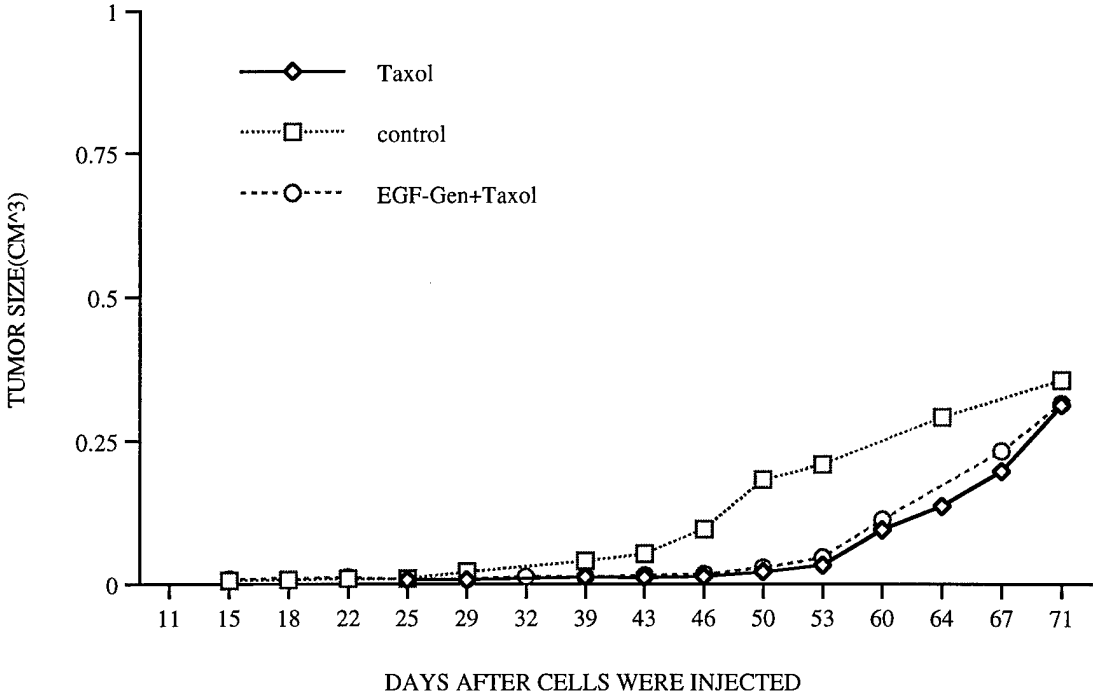


This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with Adriamycin on day 2.



This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with Cytoxan on days 2 and 3.

5/24/99 MDA Taxol DATA



This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with Taxol on days 2-4.